MOLECULAR DETECTION OF PATHOGENS IN SOUTHERN BLUEFIN TUNA

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Declarations by the Author

Statement of Originality

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The research associated with this thesis abides by the international and Australia codes on animal experimentation. Animal ethics permits were obtained from the University of Tasmania Animal Ethics Committee for all aspects of the project where experiments involved live animals (Permit number 0013175)

Signed: Date: 8th February 2017

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Abstract

Within the Australian fish production industry the ranching of Southern bluefin tuna (*Thunnus maccoyii*) (SBT) continues to be one of the most profitable. As culture practices become more efficient, the requirement for efficient methods of pathogen detection and quantification are also of increasing importance. In an effort to better understand the impact of ranching on pathogen prevalence, as well as provide tools for rapid detection, this research aimed to elucidate variability in pathogen prevalence in wild versus ranched SBT through the development of quantitative PCR methods and application of 16S rRNA amplicon sequencing. The first three research chapters focus on the detection of blood flukes (Digena: Aporocotylidae) from genus *Cardicola,* specifically *C. forsteri*, *C. orientalis* and *C. opisthorchis*; including development and application of a qPCR assay to quantify each species from organ and environmental samples. The final research chapter details the bacterial diversity in SBT spleen using universal bacterial 16S rRNA primers to amplify the V1-3 region and amplicon pyrosequencing.

The 2013 SBT ranching season marked the introduction of the anthelmintic called praziquantel (PZQ), which has also been used to mitigate *C. orientalis* and *C. opisthorchis* infections in juvenile Pacific bluefin tuna in Japanese culture systems. Both wild and ranched SBT (at harvest) were sampled annually for three consecutive years starting in July 2013. A hydrolysis probe-based qPCR assay was designed and validated to detect and quantify *C. forsteri, C. orientalis,* and *C. opisthorchis* ITS2 rDNA. The assay was applied to quantify *C. forsteri* and *C. orientalis* DNA in heart and gill, which documented a significant annual variability of *C. forsteri* infection in ranched SBT hearts. No *C. orientalis* was detected in 2014 and 2015 ranched SBT,

whereas *C. forsteri* was found in at least 95% of fish examined in all three years. This was an important yet unexpected finding given *C. orientalis* was significantly more prevalent than *C. forsteri* in 2011/12 harvests. Lastly, this chapter reports the first detection of *C. orientalis* in wild SBT.

The newly developed hydrolysis probe-based qPCR assay was utilized to examine the prevalence of *C. forsteri* and *C. orientalis* ITS2 rDNA in 1995 and 2004 archival formalin-fixed paraffin embedded (FFPE) SBT heart samples. Prior to the examination of archival samples, five FFPE DNA extraction methods were compared, consisting of with and without xylene deparaffinization, application of 1 h 90°C incubation post proteinase K incubation, a QIAamp DNA mini kit® (Qiagen) and a TRIzol reagent based protocol. In addition, extended proteinase K incubation at 37°C, 72 h instead of 24 h, was examined and resulted in a significant increase in *C. forsteri* ITS2 rDNA. The inclusion of a 90°C h sample incubation step post proteinase K incubation resulted in a 100-fold increase in DNA yield compared to without and was significantly higher than when using a Qiagen QIAamp FFPE kit. This retrospective examination documented the presence of *C. orientalis* in 1995 FFPE SBT heart, preceding previous published presence in SBT in 2008 samples.

C. forsteri and *C. orientalis* ITS2 rDNA was detected in sea water samples from sea cages alongside commercial harvest and control locations north and south of the cages. Real-time qPCR methods using SYBR green nucleic acid dye was used in the first year, and the hydrolysis probe-based qPCR assay in the second two years. Of three consecutive sampling years, *C. forsteri* and *C. orientalis* ITS2 rDNA was only detected in the first, despite qPCR examination of SBT organs documenting the presence of both blood fluke species in the all the years examined. Samples from the control North location (3.5 km North of cages containing SBT) had

significantly lower levels of *C. forsteri* and *C. orientalis* ITS2 rDNA in the water compared to cages and control South location (2 km South of cages containing SBT). Significantly more *C. orientalis* ITS2 rDNA (M = 4885.08, SD = 7328.71) was detected compared to *C. forsteri* (M = 232.67, SD = 337.82) in samples collected on 29th and 30th of August, 2012.

Culture independent PCR was applied to spleen of wild (n = 10) and ranched (n = 10) SBT as part of sample preparation for an evaluation of bacterial diversity using 16S rDNA tag-encoded pyrosequencing. The most abundant phylum among wild and ranched fish was *Proteobacteria* (94.41% ±0.041) followed by *Acidobacteria* (4.71% ±0.035), *Cyanobacteria* (0.49% ±0.007), and *Firmicutes* (0.24% ±0.005). Both *Acidobacteria* and *Cyanobacteria* were significantly more abundant in ranched compared to wild SBT ($p< 0.05$) spleen. *Bacteroidetes* was significantly more abundant in wild SBT ($p < 0.0001$) and it was not detected in samples from ranched SBT. The top 18 most abundant genera constituted 99.2% of the spleen microbiota. These included *Bosea* (66.99%), *Phyllobacterium* (22.73%), *Edaphobacter* (2.74%), *Methylobacterium* (2.03%), *Propionibacterium* (2%), *Bradyrhyzobium* 0.69%), *Ochrobactrum* 0.52%), *Mesorhizobium* (0.31%), *Pseudoalteromonas* (0.21%), *Corynebacterium* (0.19%), *Acinetobacter* (0.19%), *Chelatococcus* (0.14%), *Burkholderia* (0.1%), *Staphylococcus* (0.1%), *Psychrobacter* (0.08%), *Yersinia* (0.06%), *Enterobacter* (0.06%), and *Pseudomonas* (0.05%). Although *Edaphobacter* and *Pseudoalteromonas* were present in both groups, they were significantly more abundant in ranched SBT spleen ($p < 0.05$). On average the SBT spleen microbiota had 148.9 (±35.4) operational taxonomic units (OTUs) (min = 109; max = 252) at 97% sequence similarity. No significant difference in alpha diversity indices was found. PCoA plot based on weighted UniFrac distance as a measure of beta diversity showed no visual difference between wild and ranched SBT spleen in the community structure. No distinct difference in bacterial communities

between wild and ranched fish may suggest that the documented diversity described in this thesis represents the baseline for SBT.

The molecular detection methods described and applied in this thesis as well as the insight to bacterial diversity in SBT spleen have given a unique understanding of the presence of pathogens and will play a significant role in the sustainable ranching and management of this critically endangered bluefin tuna species.

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Explanatory note regarding thesis structure

Chapters 2, 3 and 5 are drafts of publication manuscripts at the time of submission. Hence some repetition of material between the introductory sections of the chapters has occurred. The references for each chapter can be found in the cumulated bibliography starting on page 88.

The Harvard referencing style has been used for this thesis.

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1. General Introduction

1.1. Bluefin tuna

Thunniform swimming, regional endothermy, elevated metabolic rate and frequency-modulated cardiac output are functional differences distinguishing tuna from other fish (Graham and Dickson, 2004, Fitzgibbon et al., 2008). These key advantages allow tuna expansion into high latitudes and to oceans previously thought beyond their scope (Graham and Dickson, 2004). Tuna's unique versatility and distinguishing differences have captured the curiosity of researchers for decades. Apart from slender tuna (*Allothunnus fallai)*, all tuna spawn in warm waters (Graham and Dickson, 2004, Cribb et al., 2000), which have shown to be geographically restricted, thereby putting them at risk of population collapse under excessive fishing pressure (Collette et al., 2011). Pacific bluefin tuna (*Thunnus orientalis*) (PBT), Atlantic bluefin tuna (*Thunnus thynnus*) (ABT) and Southern bluefin tuna (*Thunnus maccoyii*) (SBT) are highly lucrative commodities worldwide with Japan being the primary consumer (Allen, 2010, Collette et al., 2011). Per kilogram, bluefin tuna is among the most expensive fresh seafood in the world, with a record sale of $1M AUD for a single fish recorded at the Tsukiji fish market in Japan, 2013 (Collette et al., 2011). Such lucrative income puts wild bluefin tuna stocks at risk of overfishing and exploitation, with PBT population currently rated as vulnerable and ABT as endangered and SBT stocks as critically endangered (Collette et al., 2011).

The Japanese long line fishery for SBT started in the early 1950s, and within the following 10 years annual catch rates had increased to 80,000 tons per year (Allen, 2010). In Australia, the purse seine fishery for SBT began around the same time, exceeding 20,000 tons per year in the late 1980s (Allen, 2010). In 1996, the International Union for Conservation of Nature (IUCN) listed SBT as critically endangered and annual quotas were set in place to support wild populations to recover.

In an effort to maximize production while abiding by the restrictive bluefin tuna quotas, Japan, Australia and Mediterranean countries began ranching tuna. Ranching refers to the practice in which wild bluefin are caught and transferred to sea-cages until harvest.

In the Mediterranean tuna ranching started in Spain in 1985, rapidly expanding to 11 countries in 2007 with an annual ranching potential of 56,842 tons, of which Japan absorbed 90% (Figure 1). The Japanese demand for tuna has been the driving force behind the global tuna industry.

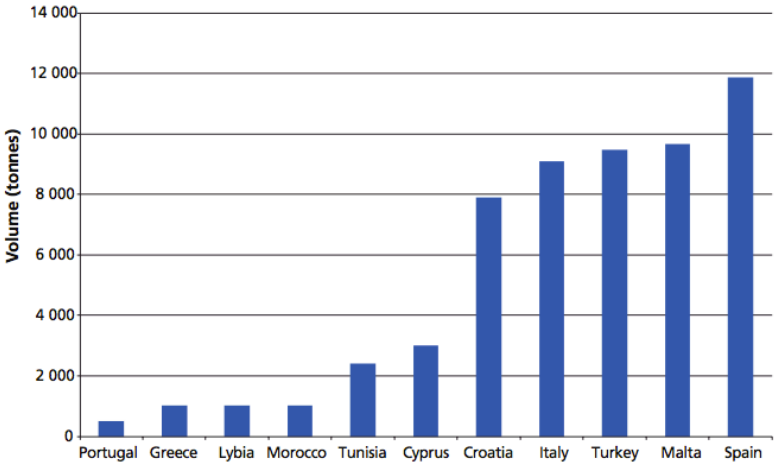

Figure 1 Mediterranean bluefin tuna ranching potential country capacity
Figure source: (Ottolenghi, 2008)

PBT ranching is very common in Japan and researchers at Kinki University have successfully closed the life cycle for this species in a closed aquaculture system (Sawada et al., 2005). Providing optimum nutrition ensures reproductive success, and plays an important role in the establishment of healthy brood stocks, i.e. production of high quality eggs and larvae (Mourente and Tocher, 2009, McKeever and Rege, 1999, Leef et al., 2012). Maximizing survival during the first 10 days post hatching is challenging, where cannibalism during larval and juvenile stages, as well as collision problems caused 100% mortality in juvenile PBT (Sawada et al., 2005, Honryo et al., 2014). The addition of night-time lighting following transfer to sea cages increased

survival rates to 96% by preventing mass death due to collision and contact with cage walls, which in turn was a significant improvement from mortality seen with no night-time lighting (Honryo et al., 2014). Mortalities of PBT during the juvenile growth stage were further reduced with the stabilization of water temperatures, where a consistent temperature ranging between 15-25°C significantly reduced juvenile PBT mortalities (Tsuda et al., 2012).

Ranching of SBT was first introduced in Australia in 1991 and has turned into a highly lucrative undertaking, making up more than $130 million in annual exports, which has made the tuna ranching industry one of Australia's most valuable seafood sectors (ABARES, 2013). The season operates between December and August when the fish migrate along the continental shelf (see Figure 2 for migratory routes) in the Great Australian Bight (Kirchhoff et al., 2011a, Dennis et al., 2011a). Two to four year old SBT are caught using purse seiners. They are then moved to tow pontoons, towed to Port Lincoln, and transferred to sea cages (Kirchhoff et al., 2011a). With a limited annual catch quota of 5665 tons for the 2016-17 season (CCSBT, 2017), regular monitoring of SBT health supports efficient production and industry growth while observing conservation regulations.

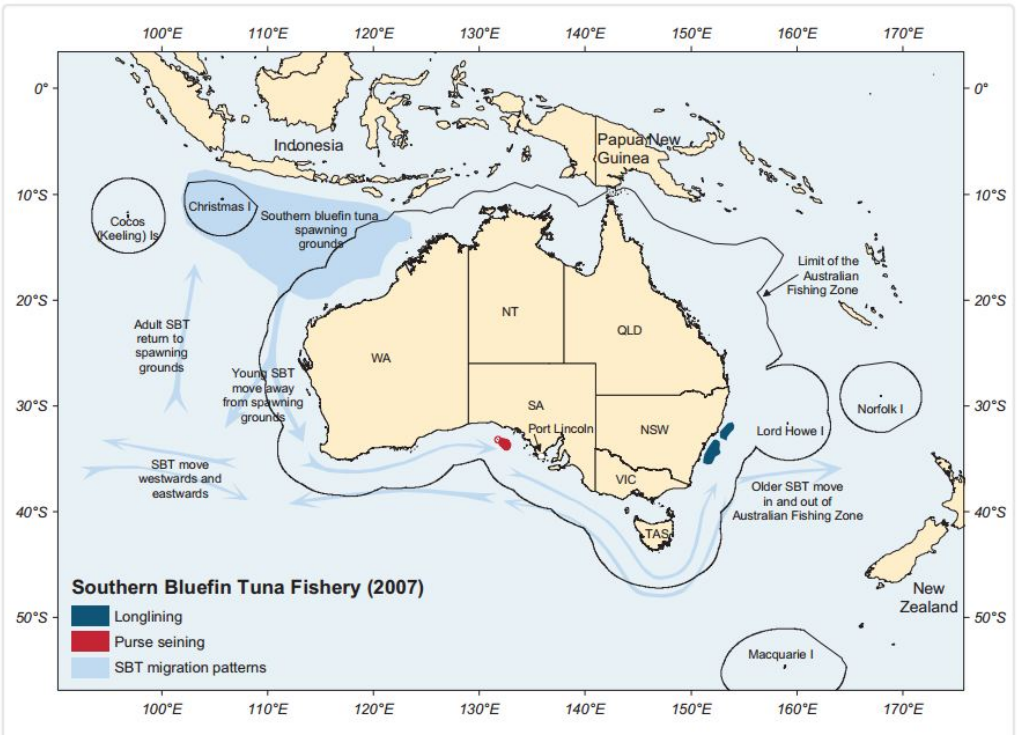

Figure 2 Spawning grounds, migration patterns (arrows) and fishing grounds for SBT around Australia

Illustration source: (AFMA, 2007)

1.2. Blood flukes affecting bluefin tuna

Infection with blood flukes (*Cardicola forsteri* and *C. orientalis*) is the main factor affecting the health of commercially ranched SBT in South Australia, with rates of infection reaching up to 100% in the first two months of being transferred to sea cages (Aiken et al., 2008, Hardy-Smith et al., 2012). Table 1 illustrates the classification of the genus *Cardicola*, which is a member of the Aporocotylidae fish parasites, which encompass 33 genera, such as *Skoulekia*, *Selachohemecus,* and *Sanguinicola* (Gibson, 2014). To date, 31 different species from genus *Cardicola* have been documented in various hosts and in various life stages.

Table 1 Classification of *Cardicola* spp.

Biota	Kingdom	Phylum	Class	Subclass	Order	Suborder	Superfamily	Family
	Animalia	Platyhelminthes	Trematoda	Digenea	Diplostomida	Diplostostomata	Schistosomatoidea	Aporocotylidae

As described by Ogawa (2010) and Palacios-Abella (2015), there are four species of *Cardicola* specific to bluefin tuna: *C. forsteri*, *C. orientalis*, *C. opisthorchis* and one remains to be named (Ogawa et al., 2010, Ogawa et al., 2011, Palacios-Abella et al., 2015). *C. orientalis* (Ogawa et al., 2010) was initially thought to only infect PBT, however a study conducted in the year following species description revealed that it also can be found in ABT and SBT (Polinski et al., 2013a, Palacios-Abella et al., 2015). While *C. forsteri*, *C. orientalis*, *C. opisthorchis* have been detected in ranched PBT and ABT, a recent study conducted by Palacios-Abella (2015) also found a forth (unnamed) species in ranched ABT (Forte-Gil et al., 2016, Shirakashi et al., 2016b, Palacios-Abella et al., 2015). Additionally, *C. opisthorchis* and the unnamed species are yet to be reported from SBT.

1.3. *Cardicola* spp. life cycle

To maximize tuna growth during the ranched period it is important to be familiar with the life cycle of the flukes as well as the seasonal variations (Bullard and Overstreet, 2002c). Helminth parasite life cycles generally include a primary host where sexual reproduction occurs, and either one or two intermediate hosts where asexual reproduction can also occur (Bell and Burt, 1991, Adams et al., 1997). Aporocotylids, or fish blood flukes have one intermediate host (Adams et al., 1997). Terebellid polychaetes are known as the intermediate host for *C. forsteri, C. orientalis* and *C. opisthorchis* (Table 2). *C. forsteri* has been documented in *Amphitrite* spp., *Longicarpus*

modestus and *Reterebella aloba*, whereas *C. orientalis* and *C. opisthorchis* life cycles stages been discovered in *Nicolea gracilibranchis* and *Terebella* spp., respectively.

Table 2 Documented terebellid polychaete intermediate hosts of *Cardicola forsteri, Cardicola orientalis* and *Cardicola opisthorchis*

Terebellid polychaete	*C. forsteri*	*C. orientalis*	*C. opisthorchis*	Source
Amphitrite spp.	X			(Shirakashi et al., 2016a)
Nicolea gracilibranchis		X		(Shirakashi et al., 2016a)
Longicarpus modestus	X			(Cribb et al., 2011)
Terebella spp.			X	(Sugihara et al., 2014)

Generally, blood fluke cercariae penetrate the fish through the gills, skin, eye or alimentary tract and find their way to the heart through the cardiovascular system where they mature to their adult stage (Bullard and Overstreet, 2002c). Adult stage blood flukes can be found in the heart or blood vessels in the gills, kidney and spleen; where thin shelled eggs are released into the bloodstream and become trapped in the microvasculature of the highly oxygenated gills (Bullard and Overstreet, 2002c). As with other species of blood fluke, once the *C. forsteri* egg hatches the miracidia leave the fish in search of its intermediate host (Cribb et al., 2011). Once mature, *C. forsteri* cercariae leave the intermediate host, possibly killing it (Rough, 2000, Colquitt et al., 2001c). Due to the short, simple tail, it is speculated that *C. forsteri* cercariae are not active swimmers and rely on marine currents for distribution in their 24-48 h lifespan (Cribb et al., 2011, Sugihara et al., 2014). The life cycle of *C. forsteri* is shown in Figure 3.

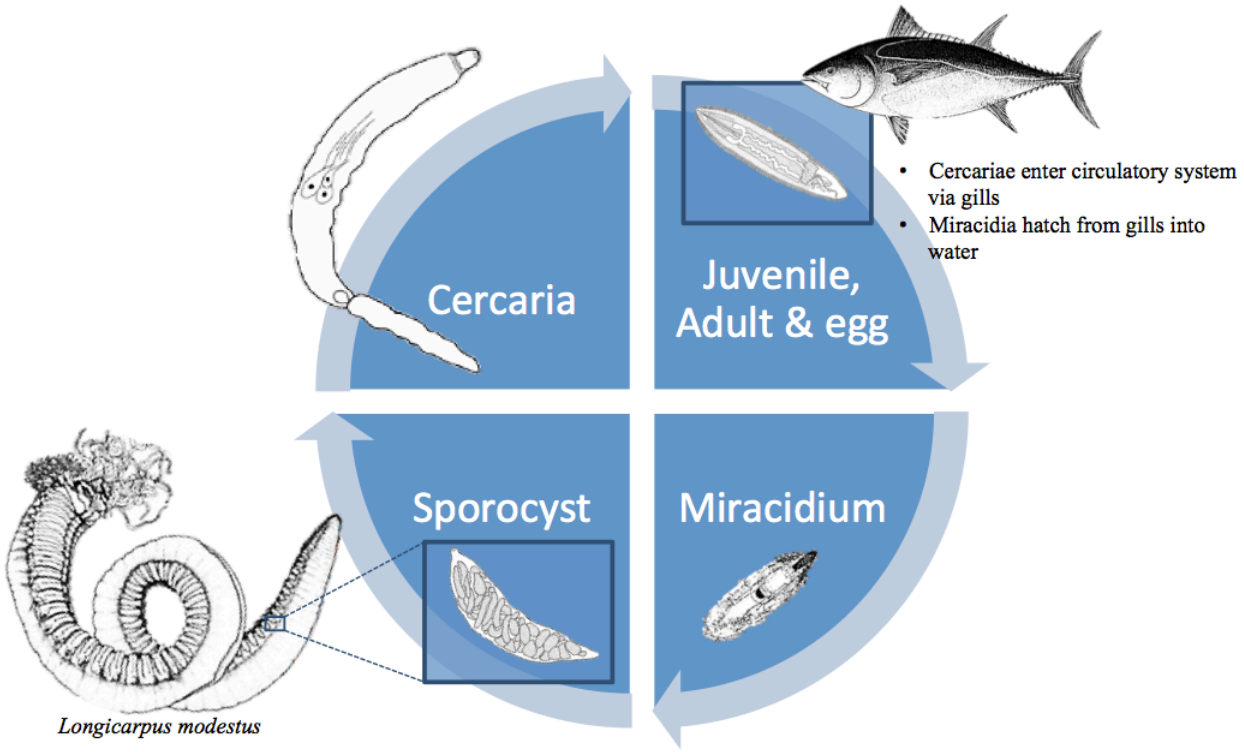

Figure 3 The life cycle of *Cardicola forsteri*. Illustrations source: (Beesley et al., 2000, Collete and Nauen, 1983, Cribb et al., 2011, Shirakashi et al., 2013)

1.4. *C. forsteri* and *C. orientalis* in SBT

Studies conducted prior to the introduction of PZQ for the treatment of blood flukes in SBT surveyed the prevalence of adult *C. forsteri* in the hearts of SBT throughout the 6 months they are held in sea cages (Ottolenghi, 2008, Aiken, 2009). Once tuna were transferred to ranching pens, there was a rapid increase in prevalence, with a peak of 100% infection and an average of 27 adult flukes per tuna heart within 2 months. In the following months *C. forsteri* prevalence slowly decreased to 35% (Aiken et al., 2006). In the heart, adult *C. forsteri* caused hypertrophy of the cardiac spongiosa, that could reduce the stroke volume, thereby requiring an increased heart rate (Colquitt et al., 2001a). This stress may cause anoxia or hypoxia in fish organs and demands for increased oxygen supply, which can lead to heart failure (Colquitt et al., 2001a).

Blood fluke eggs can be predominantly found in the second gill arch of SBT (dos Santos et al., 2012b), although they have also been detected in heart, kidney, liver, and spleens of infected fish (Colquitt et al., 2001a). Histopathological examination for *C. forsteri* eggs in ranched SBT heart showed that the eggs were encapsulated by granulomatous reactions made up of epithelioid cells and lymphocytes surrounded by fibroblasts and fibrocytes in older lesions (Aiken, 2009).

1.5. Detection of *C. forsteri* and *C. orientalis*

Development of DNA sequencing has allowed researchers to identify and document *Cardicola* spp. even if found in low numbers or only one stage of the life cycle (Polinski et al., 2013b, Caldas et al., 2012, Polinski et al., 2013a). Traditional diagnostic tools for identifying *Cardicola* spp. in SBT relied on visual confirmation of parasites in gill and heart flushes (Kirchhoff et al., 2011b, Aiken et al., 2006, Shirakashi et al., 2013) or histological sections of heart and gill (dos Santos et al., 2012a). Heart and gill flushes are time-consuming and require processing within 48 h of collection, consequently limiting the maximum number of hearts that can be evaluated.

Heart flush microscopy is a method where the fish heart is cut in half, horizontal and vertical sections are cut in each respective half about 1cm apart with a new scalpel blade and are then washed with a 50:50 mixture of saline and tap water (Colquitt et al., 2001c, Aiken et al., 2006). Once the run-off saline is clear the mixture is split into 3-5 petri dishes and scanned with a dissection microscope to reveal adult blood fluke. In addition, adult flukes and eggs can be identified using gill and heart histology, where a piece of organ is fixed, embedded, sectioned, H&E stained and examined at 10 to 40 times magnification. Further, the total number of eggs in

fresh and frozen whole gill filaments of juvenile fish can be attained using microscopic examination of gill filament mounts (Shirakashi et al., 2012b).

Polymerase Chain Reaction (PCR) is a widely used method for the detection of various pathogens including parasitic infections (Aiken et al., 2007a, Forte-Gil et al., 2016, Weiss, 1995). Both in ABT and PBT *Cardicola* spp. research, standard PCR has been used for species-specific prevalence analysis (Forte-Gil et al., 2016, Sugihara et al., 2015); though highly specific it is not quantitative. Real time qPCR permits quantification of the target analyte and is a standard for the rapid diagnosis of many diseases (Altinok and Ilknur, 2003, Netto et al., 2003, Cunningham, 2002), particularly where accurate and rapid detection at low levels of infection is a means of early diagnosis not possible using microscopy methods (Perandin et al., 2004, Cohen et al., 2013). Today, qPCR methods are frequently used to detect parasitic infections such as malaria in humans and *Myxobolus cerebralis* in rainbow trout (*Oncorhynchus mykiss*) (Perandin et al., 2004, Cohen et al., 2013, Cavender et al., 2004).

Methods for the detection of *C. forsteri, C. orientalis* and *C. opisthorchis* in Bluefin tuna have been researched in great detail (Belworthy, 2012, Polinski et al., 2013a, Polinski et al., 2013b, Ogawa et al., 2010, Shirakashi et al., 2013, Sugihara et al., 2016). The use of real-time quantitative polymerase chain reaction (qPCR) has shown to be more sensitive and precise at identifying the presence of *C. forsteri* and *C. orientalis* than previously used methods, such as histology and microscopic evaluation of heart flushes (Polinski et al., 2013b). There are numerous chemistry options available for quantitative PCR, dsDNA binding dye and hydrolysis probe-based assays being the two most popular and widely used chemistries. The former detects PCR product, while the latter uses a specific probe to detect target analyte as it accumulates during PCR. Cost, sensitivity and reproducibility of dsDNA binding dye based detection is

variable, though cheaper than hydrolysis probe qPCR detection which in turn is known to be highly specific, reproducible and able to detect low DNA copies. By targeting the ITS2 region of the rDNA for PCR amplification a sensitivity limit of 3-10 genomic copies can be achieved without cross species amplification (Polinski et al., 2013b, Belworthy, 2012).

In addition, emergence and validation of highly sensitive qPCR methods has opened the doors to retrospective examination of archival formalin-fixed paraffin embedded (FFPE) SBT organs. *C. orientalis* was described in PBT in 2010 (Ogawa et al., 2010) and its 28S rRNA, 5.8S rRNA and ITS2 rRNA gene sequences were published in 2012 (GenBank: AB742427.1) (Shirakashi et al., 2012b). *C. orientalis* was subsequently detected in SBT in 2013 (Shirakashi et al., 2013). qPCR examination of 2008 FFPE SBT heart and 2008 serum (Polinski et al., 2013a) showed *C. orientalis* was significantly more prevalent than *C. forsteri* in 2008. *C. forsteri* was previously thought to be the only blood fluke from genus *Cardicola* infecting SBT (Cribb et al., 2000, Colquitt et al., 2001c, Deveney et al., 2005).

FFPE samples are of great value as they allow retrospective epidemiology research. For example, a retrospective study of FFPE tumour samples from patients with prostate cancer allowed researchers to explore the prognostic value of RNA expression signature resulting from cell cycle proliferation (Cuzick et al., 2011). Similarly the availability of archival FFPE samples enabled a retrospective study of leproid granulomas in canines, focusing on clinical signs, histopathology and molecular characterization (Conceição et al., 2011).

A previous study that examined the effects of fixatives used for *Neoparamoeba perurans* DNA extraction efficacy and quantity from salmon gills, found seawater Davidson's and PAX-gene® provided good preservation of DNA and tissue morphology, thereby allowing both histological

and molecular analysis from the same sample (Cadoret et al., 2013). To date no comparison of DNA extraction methods for subsequent qPCR *C. forsteri* and *C. orientalis* detection has been conducted on FFPE material.

Further, hydrolysis probe-based qPCR is frequently used for the detection of pathogens in environmental samples. Molecular detection of parasites (Audemard et al., 2004, Hallett and Bartholomew, 2006), bacteria (Lee et al., 2006, Shannon et al., 2007) and viruses (Miagostovich et al., 2008) from water can provide valuable information regarding their presence, quantity and distribution allowing for preventative measures to be taken (Girones et al., 2010). The detection of parasites during their waterborne infective life stages, particularly ones known to jeopardize the productivity of marine aquaculture systems, has been a valued non-invasive method of pathogen identification (Audemard et al., 2004, Hung and Remais, 2008).

1.5.1. Treatment and management of tuna infection with blood flukes

As with any type of farming, it is in the tuna farmers' best interest to prevent disease spread and to provide rapid treatment upon detection, thereby ensuring the highest possible yield of harvest at the end of the season. For example, in ranched SBT systems it is of utmost importance to maximize weight gains in the 6 months the fish are kept in sea cages (Volpe, 2005). Any form of stress will jeopardize fish growth; therefore treatment and prevention of infections with blood flukes are vital. A recent study analyzed the effectiveness of four anthelmintics, both *in vivo* and *in vitro*. PZQ caused a significant reduction *in vitro* in the mean number of responsive adult flukes after 48 hours of treatment at a concentration of 0.125 µg/mL (Hardy-Smith et al., 2012, Ishimaru et al., 2013). Praziquantel has been deemed a highly effective and safe method of

controlling blood fluke populations in farmed PBT (Ishimaru et al., 2013, Shirakashi et al., 2012a). The drug does not seem to affect eggs as viable miracidia were observed throughout a study conducted by Shirakashi et al. (2012) which examined hatchery produced juvenile PBT naturally infected with *C. orientalis* and *C. opisthorchis*.

A recent study conducted by Polinski et al (2014) provided preliminary evidence of transcriptional immunomodulation by PZQ in SBT blood cell and anterior intestinal explant *in vitro* cultures (incubation using L-15 supplemented growth media), thereby suggesting potential as an immunostimulant inferring a stronger fish immune response to blood fluke infection. Independent of parasite antigen presence, PZQ was shown to induce a heighted immune signaling in tissue/organ cultures. This particular study observed an elevation of IL-8 chemokine gene expression as well as both T and B cell-specific transcriptional signaling. This in turn suggests that an immune response may contribute to the *in vivo* effectiveness of PZQ treatment of blood flukes (Polinski et al., 2013c).

1.6. Other pathogens of concern for SBT

While blood flukes from the genus *Cardicola* are the primary threat to the health of ranched SBT, previous studies have explored the microbial characteristics of bluefin (Roberts and Agius, 2008, Valdenegro-Vaga et al., 2013) using culture methods. Bacterial isolates have been recovered from spleen, kidney and gill of harvested and moribund/dead SBT (Valdenegro-Vaga et al., 2013). Although more culturable isolates were obtained from gill, bacteria previously linked to mortalities in gilt-head bream (*Sparus aurata*), seabream (*Abramis brama*), seabass (*Dicentrarchus labrax*) and sole (*Solea solea*) mariculture systems in Mediterranean countries of

Europe and hybrid striped bass (*Morone saxatilis*) in the USA (Toranzo et al., 2005) have been isolated from spleen and kidney of SBT (Valdenegro-Vaga et al., 2013).

In a previous, culture-based study, *Photobacterium damselae* subsp. *damselae* was the dominant subspecies identified in SBT spleen, and has shown to cause systemic infections in other fish species (Gauger et al., 2006, González et al., 2004, Osorio et al., 2000, Pedersen et al., 2008). *Photobacterium damselae* subsp. *piscicida*, the causative agent of Pasteurellosis, was isolated from the spleen of harvested SBT (Valdenegro-Vaga et al., 2013). This species has been linked to mass mortalities of ABT in the Mediterranean culture systems (Mladineo et al., 2006). It is speculated that under conventional rearing conditions all bluefin tuna are able carry *P. damselae* subsp. *piscicida* and show no clinical signs of infection (Munday et al., 2003), whereas mortalities in turbot (*Scophthalmus maximus*) as a result of this bacteria have been linked to sub-optimal rearing temperatures surpassing 18-20°C (Toranzo et al., 2005). In farmed ABT the highly abundant digenean *Didymocystis wedli* infection resulted in the formation of cysts in the fish intestinal tract, which in turn have shown to cause secondary bacterial infections (Mladineo, 2006). The link to parasite presence, as well as microbiological characteristics and potential variation between wild and ranched tuna remains to be fully explored (Fernandes et al., 2007a, Fernandes et al., 2007b).

The investigation of microbiomes of internal organs and systems previously thought to be sterile in a healthy animal is a relatively new field. In humans, indigenous microbiotas have been evaluated in various internal organs (Abu-Shanab and Quigley, 2010, Beck et al., 2012, Urbaniak et al., 2014, Wolfe et al., 2012, Jiang et al., 2015, Bik et al., 2006), and abnormalities in these

respective communities have been linked to severe health conditions (Amar et al., 2013, Turnbaugh et al., 2006). This may also be the case in the indigenous microbiotas of fish organs. High-throughput next-generation pyrosequencing is a sequencing technology that does not rely on media-based culture of bacteria by targeting the hyper-variable regions of 16S rRNA gene in bacteria (Benítez-Páez et al., 2013, Kakizaki et al., 2012).

1.7. Research objectives

The overall objective of this thesis was to develop molecular techniques for the detection and quantification of SBT pathogens with a particular focus on blood flukes from the genus *Cardicola*. Another aspect investigated in this thesis was bacterial diversity in the spleen of apparently healthy SBT based on molecular analyses.

Specific aims were to:

- Validate a new qPCR protocol for detection of *C. forsteri, C. orientalis,* and *C. opiosthorchis.* The assay was applied to determine inter-annual variability in prevalence and intensity of *C. forsteri* and *C. orientalis* in wild and ranched SBT gill and heart.
- Determine DNA extraction efficacy from FFPE SBT, and application of previously validated hydrolysis probe-based qPCR assay to interrogate 1995 and 2004 samples for the presence of *C. orientalis*.
- Determine the presence of *C. forsteri* and *C. orientalis* DNA in seawater using qPCR.
- Apply high-throughput 16S rDNA pyrosequencing to evaluate bacterial diversity in spleen of wild and ranched SBT.

2. Annual variability of *Cardicola forsteri* and *Cardicola orientalis* infection in ranched and wild Southern bluefin tuna (*Thunnus maccoyii*)

2.1. Introduction

The Southern bluefin tuna (SBT) is a highly profitable and important species to the Australian seafood industry (ABARES, 2013). In recent decades the SBT fishery has moved toward ranching to augment total saleable yields while abiding by annual wild catch quotas (AFMA, 2007). Wild juvenile SBT are caught in the Great Australian Bight and are towed in cages to farm sites near Port Lincoln (Kirchhoff et al., 2014, Kirchhoff et al., 2011b, Nan et al., 2016). The SBT are transferred to grow-out sea cages where they are fed daily to maximize growth before harvest, this process generally increases individual fish weight by approximately 10-20kg over 6 months (Volpe, 2005). The primary health factor affecting ranched SBT in Australia is infection by blood flukes from the genus *Cardicola* (Colquitt et al., 2001b, Cribb, 2000, Munday et al., 2003). Seasonal mortalities of SBT have been attributed to infections with *Cardicola forsteri* and *Cardicola orientalis* (Cribb, 2000, Deveney et al., 2005, dos Santos et al., 2012a). Similarly in Japanese cultured Pacific bluefin tuna (PBT), *C. orientalis* infection is one of the main causes of mortality in hatchery-reared juveniles (Shirakashi et al., 2012b, Sugihara et al., 2016). *C. forsteri, C. orientalis* and *C. opisthorchis* infection is also the primary health concern in sea-cage reared Atlantic bluefin tuna (ABT), though no significant difference in level of blood fluke infection have been documented between wild and ranched fish (Forte-Gil et al., 2016).

At Kinki University in Japan, more than 50% of juvenile PBT mortality has been associated with blood fluke infection, with infection prevalence reaching 100% within 3 months of transfer to sea cages (Ishimaru et al., 2013). Since 2012 the anthelmintic praziquantel (PZQ) has been used as an effective treatment method of controlling blood fluke infection in farmed and ranched tuna (Ishimaru et al., 2013, Shirakashi et al., 2012a). A study at Kinki University investigating PZQ doses found that when administered at either 15 and 150 mg kg^{-1} bodyweight (BW) for three

consecutive days respectively it eradicated adult worms within 8 days post treatment (Shirakashi et al., 2012a). PZQ has been used in the Australian SBT industry since early 2013, since then annual mortality has been reported at less than 1%, much lower than 10-15% in previous years (Polinski et al., 2014). This study examined the first three consecutive years after the introduction of PZQ treatment.

Research concerning blood fluke detection and annual variation in prevalence has historically relied on a limited suite of methods including histology and saline heart flushes (Bullard et al., 2004, Colquitt et al., 2001c, Cribb, 2000, Dennis et al., 2011a, Dennis et al., 2011b, Deveney et al., 2005, Kirchhoff et al., 2012, Santos et al., 2012, Shirakashi et al., 2013, Kirchhoff et al., 2014). Histological analysis permits the quantification of eggs and granulomas in hematoxylin and eosin (H&E) stained organ sections without the ability to identify which species is being detected. Heart flushes are used to quantify adult blood flukes in the whole heart (predominantly *C. forsteri* adults in the heart), though differentiation of *C. forsteri* and *C. orientalis* is possible, this requires time and experience (Aiken, 2009). *C. forsteri* and *C. orientalis* can be detected and differentiated to species using PCR analysis, even in samples containing trace amounts of the parasite's DNA thereby surpassing every other method with its detection sensitivity, specificity and speed (Polinski et al., 2013b, Polinski et al., 2013a).

This study documents annual variability of *C. forsteri and C. orientalis* infection and prevalence in wild and ranched SBT using a highly sensitive and specific quantitative PCR (qPCR) to analyze samples from three consecutive annual harvests following the introduction of PZQ in 2013.

2.2. Materials and methods

2.2.1. Ethics statement

All wild SBT sampling procedures were approved by the University of Tasmania Animal Ethics Committee (A0013175).

2.2.2. Sample collection and processing

Samples of organs from ranched SBT were collected at the same time of each year (early July), during commercial SBT harvests with the same company which used the same husbandry practices over those years, in Port Lincoln (Coordinates: 34°41’26.6"S 135°57’37.8"E), South Australia in 2013 (n = 50), 2014 (n = 60), and 2015 (n = 40). Wild SBT were caught between January and May 2014 (n = 30) and 2015 (n = 16) by trolling in waters surrounding Pedra Branca (Coordinates: 43°51’00″S 146°58’12″E), Tasmania.

A 5 cm x 5 cm section of filaments from the center region of the second left gill arch was placed in an individual sealed bag and stored on ice (dos Santos et al., 2012a, Shirakashi et al., 2012b). A 0.5 cm^3 piece of heart (taken from near the apex of the ventricle) and additional gill filaments from the center region of the second left gill arch was preserved in 1 mL RNA preservation reagent (4M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA, pH 5.2) and placed on ice for subsequent DNA extraction. The remaining heart was placed in a 450 mL plastic container and kept on ice until heart flush processing upon arrival at the laboratory 3–4 h later.

For histology, a 1 cm x 1 cm piece of the 5 cm x 5 cm piece of gill was placed in 10% neutral-buffered formalin (NBF). A thin section of heart 0.5 cm x 0.5 cm comprised of both compacta and spongy muscle from near the apex was placed in 10% NBF for histology. After 24 h

fixation, the samples were transferred to 70% ethanol. The samples were dehydrated using a sequence of alcohols at increasing concentrations (80%, 95% and 100%), embedded in paraffin, sectioned at 5 μm and then stained with (H&E) using standard methods (Brown, 2002). Gill samples were decalcified in Rapid decalcifying fluid (Australian Bio-stain, VIC, Australia) for 2 h prior to processing.

Heart flushes were conducted for quantification of adult *C. forsteri* and *C. orientalis* in each heart (Colquitt et al., 2001c, Aiken et al., 2006). A vertical incision was made, partially exposing the bulbous arteriosus, ventricle and atrium without cutting the heart in half. Vertical and horizontal cuts were made in the ventricle without cutting through the muscle. The two halves were then washed with a 50:50 mixture of sea water and fresh water, which was collected and poured into clean petri dishes (3–5 per heart) and left for 10 min to allow the red blood cells to settle. The dishes were then examined for adult flukes using a dissecting microscope and quantified.

2.2.3. Primer design

Primers (Table 3) were designed using Beacon Designer ™ 8 (Premier Biosoft, CA, USA) and Geneious ® 6 software. Primers were targeted against heterogeneous areas of the internal transcribed spacer-2 (ITS2) region of rDNA specific to *C. forsteri*, *C. orientalis*, and *C. opisthorchis* available on GenBank (Aiken et al., 2007a, Shirakashi et al., 2013, Ogawa et al., 2010, Cribb et al., 2011). Specificity of F and R primers was shown by Polinski et al (Polinski et al., 2013a).

Table 3 Oligonucleotide primers and probes used to amplify *Cardicola forsteri, Cardicola orientalis,* and *Cardicola opisthorchis* for real-time qPCR detection

Target	GenBank accession #	Name	Amplicon size	Sequence (5'- 3')
C. forsteri **(ITS2 rDNA)**	EF661575	Cfor_F Cfor_R Cfor_probe	287 bp	TGATTGCTTGCTTTTTCTCGAT TATCAAAACATCAATCGACATC HEX – CCACGACCTGAGCACAAGCCG – BHQ1
C. orientalis **(ITS2 rDNA)**	HQ324226	Cori_F Cori_R Cori_probe	191 bp	TGCTTGCTATTCCTAGATGTTTAC AACAACTATACTAAGCCACAA HEX – CACAAGCCGCTACCACAATTCCACTC – BHQ1
C. opisthorchis **(ITS2 rDNA)**	HQ324228	Copis_F Copis_R Copis_probe	272 bp	TTCCTAAATGTGTGTGCA TCAAAACATCAATCGACACT HEX – CACGACCTGAGCACAAGCCG – BHQ1

2.2.4. Nucleic acid extraction

Total nucleic acid (TNA) was extracted from 10 mg of RNA preservation reagent preserved gill and heart samples. Each sample was suspended in 500 µL extraction buffer (4 M Urea, 1% SDS, 0.2 M NaCl, 1mM Na Citrate) containing 5 µL proteinase K (Bioline, NSW, Australia) and incubated at 37°C for 1 h with a 5 s vortex every 10 minutes. Samples were then cooled on ice for 5 minutes; protein was precipitated by adding 350 µL 7.5 M ammonium acetate, 20 s vortex and centrifugation at 16,000 x g for 3 min at 18°C. TNA was subsequently precipitated from the supernatant by adding an equal volume of isopropanol containing co-precipitant pink (Bioline) followed by centrifugation at 16,000 x g for 30 min. The resulting TNA pellet was rinsed twice with 1 ml 75% ethanol and re-suspended in 35 µL nuclease-free buffered water (0.05% Triton X-100, 10 mM Tris). A 1:10 dilution of the original re-suspension was used for most detection and quantification. In samples with target analyte quantities below the limit of detection (less than 95% positive replicates) a 1:2 or 1:5 dilution of original pellet re-suspension was used.

2.2.5. Real-time PCR

A CFX Connect Real-time PCR Detection System (Bio-Rad, NSW, Australia) was used for real-time qPCR analyses. For *C. forsteri* and *C. orientalis* detection, 2× MyTaq™ HS mix (Bioline) was used in combination with 150 nM species-specific probe; 400 nM forward and reverse primer, and molecular water. Probes were labeled at the 5' end with 6-carboxy-2,4,4,5,7,7 -hexachlorofluorescein succinimidyl ester (HEX) together with a Black Hole Quencher® (BHQ1, Biosearch Technologies, CA, USA) added to the 3' terminus. Cycling conditions comprised an activation of DNA polymerase at 95°C for 3 min, followed by 45 cycles of 10 s at 95°C and 30 s

at 60°C, with relative fluorescence measured at the end of each 60°C combined annealing and extension step.

2.2.6. Validation of assay: limit of detection (LOD), limit of quantification (LOQ) and copy number calculation

The limit of detection (LOD) for each assay was defined as the lowest analyte concentration at which at least 95% of the independent biological replicates were positive. The limit of quantification (LOQ) refers to the lowest independent analyte dilution that can be reliably quantified with a mean coefficient of variance (CV) less than 35% (Armbruster and Pry, 2008). Both the LOD and LOQ for each assay were determined using 8 independently replicated dilutions of 11 target DNA copy number concentrations. Target DNA used for the each of the dilutions consisted of a synthetic double-stranded DNA named a gBlock® that was manufactured by Integrated DNA Technologies (Iowa, USA). Each of the gBlock®s contained the respective *Cardicola* spp. specific 287 bp (*C. forsteri*), 191 bp (*C. orientalis*), and 272 bp (*C. opisthorchis*) ITS2 rDNA sequences flanked by 413 bp, 333 bp and 248 bp of non-specific DNA, respectively. Each gBlock sequence was verified by the manufacturer and accurately supplied as a 200 ng quantity that was converted to a copy number per mass using a nucleotide specific DNA copy number calculator (http://www.endmemo.com/bio/dnacopynum.php).

Calculated analyte concentrations were determined using a mechanistic model termed ‘cm3’ developed by Carr & Moore (Carr and Moore, 2012) that is incorporated into the qpcR package (Ritz and Spiess, 2008) within R software v3.2.2 (Team, 2013) that was manipulated using R Studio© v0.99.902 software (Studio, 2012). The cm3 mechanistic model uses fluorescence

signals of later cycles and considers variables such as the influence of baseline adjustment errors, reaction inefficiencies, signal loss per cycle and template abundance, thereby making it independent of qPCR reaction efficiencies that are a common source of error when using existing methods based on the crossing threshold (Cq or Ct). The D0 parameter derived from the cm3 model was calibrated for each assay using a minimum of 16 independently replicated gBlock dilutions each estimated to contain 1 copy. These gBlock dilutions allowed a more precise and accurate copy number to be established when an algorithm was used that models the number of unamplified nil qPCR reactions to the amplified positive qPCR reactions using a Poisson distribution analogous to digital PCR (Rutledge and Stewart, 2010).

2.2.7. Statistical analysis

GraphPad Prism 5 (GraphPad software, CA, USA) was used to perform one-way analysis of variance (ANOVA). Tukey's posthoc test was used to determine significant difference between *C. forsteri* ITS2 rDNA between years in gill and heart. Linear regression analysis was used to determine the relationship between independent gBlock dilutions and calculated DNA copies. Correlation analysis was used to compare detected *C. forsteri* ITS2 rDNA calculated in heart/gill and adult flukes counted using heart flush microscopy in the same fish. A $P < 0.05$ was considered significant. Shapiro-Wilk's W test was used to test assumption of normality. Levene's F test was used to confirm homogeneity of variances.

2.3. Results

2.3.1. Limit of Detection (LOD) and Limit of Quantification (LOQ) for qPCR

The assay limit of quantification with a 95% confidence level was 8 copies when quantifying *C. forsteri*, 16 copies for *C. orientalis* and 25 copies for *C. opisthorchis* (Figure 4. A-C). The limit of detection, where the assay accurately detected expected ITS2 rDNA numbers was 4 copies for *C. forsteri* and *C. opisthorchis*, and 8 copies for *C. orientalis*. The coefficient of variation (CV) remained less than 35% in standard dilutions greater than the LOD in all three standard curves (Figure 4).

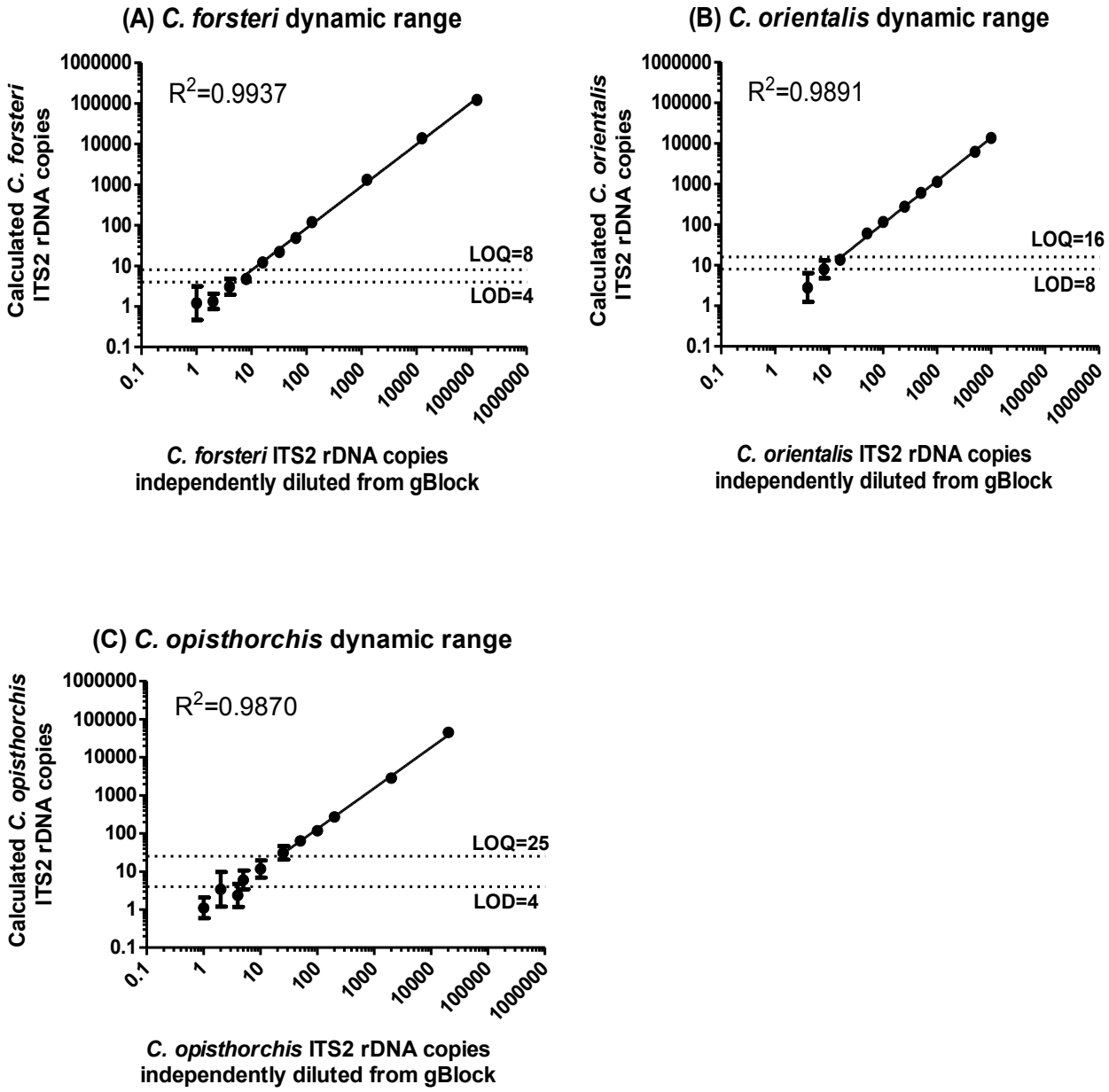

Figure 4 The dynamic range, limit of quantification (LOQ) and limit of detection (LOD) for the three *Cardicola* species-specific hydrolysis probed-based quantitative real-time PCR assays. Data were obtained using 8 independently replicated dilutions of at least 10 concentrations of a synthetic double stranded gBlock DNA standard.

2.3.2. Detection of *C. forsteri* and *C. orientalis* in SBT

In heart, *C. forsteri* was detected in 97.1% of samples in 2013, 98.3% in 2014 and 60% in 2015 using qPCR (Table 3). Being limited to visual identification of *Cardicola* spp. eggs, histological examination of heart sections of the same fish presented an egg prevalence of 10% in 2013, 73% in 2014 and 17.5% in 2015. No *Cardicola* eggs were seen in gill histology in 2013, whereas qPCR analysis of respective gills showed a 100% prevalence of *C. forsteri* and 15.7% *C. orientalis*. Histological examination of 2014 and 2015 gills, presented an egg prevalence of 68% and 5%, respectively. qPCR analysis showed a *C. forsteri* prevalence of 18.3% in 2014 and 95% in 2015 gill, and no *C. orientalis* was detected in ranched SBT heart and gill in 2014 and 2015. Of the 2014 heart samples, 20 were examined for *C. opisthorchis* using qPCR, and all were negative. No further harvest samples were examined for *C. opisthorchis* ITS2 rDNA.

Table 4 Prevalence (%) of *Cardicola forsteri* and *Cardicola orientalis* in heart flush microscopy, histological examination and qPCR analysis of heart and gill of samples collected in 2013, 2014, and 2015 SBT harvest.

	Method	**Target**	**2013** (n = 50)	**2014** (n = 60)	**2015** (n = 40)
Heart	Heart flush	Adult flukes	48%	76.6%	40%
	Histology	Eggs	10%	73.3%	17.5%
	qPCR	*C. forsteri*	97.1%	98.3%	60%
		C. orientalis	11.4%	0%	0%
Gill	Histology	Eggs	0%	68.3%	5%
	qPCR	*C. forsteri*	100%	18.3%	95%
		C. orientalis	15.7%	0%	0%

Analysis of variance followed by Tukey's posthoc analysis showed a significant difference between *C. forsteri* ITS2 rDNA in ranched SBT 2014 heart samples compared to 2013 and 2015 while there was no significant difference in gill *C. forsteri* ITS2 rDNA among the three years (Figure 5). No *Cardicola* eggs were detected via histology (heart) and qPCR ITS2 rDNA analysis (heart and gill) in wild SBT (n = 30) in 2014. Real-time qPCR analysis of 2015 wild SBT (n = 16) heart and gill, showed presence of *C. forsteri* in 3 fish (18.8%) and *C. orientalis* in 2 fish (12.5%). To the best of our knowledge this is the first detection of *C. forsteri* in wild SBT caught along the Tasmanian coast, and the first case of *C. orientalis* in wild SBT.

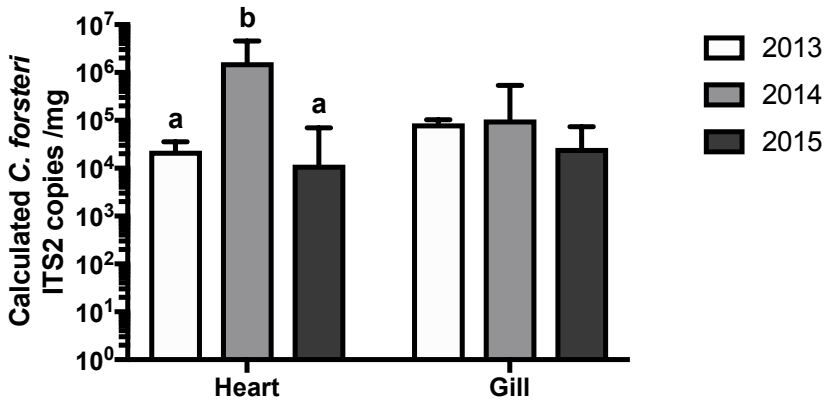

Figure 5 Calculated *Cardicola forsteri* ITS2 rDNA quantity per mg in heart and gill samples from 2013, 2014 and 2015 ranched SBT. Data shown are the mean (± 95% Cl) of all SBT heart and gill samples examined in 2013 (n = 50), 2014 (n = 60), and 2015 (n = 40). Different letters denote significant differences at $P < 0.05$.

Correlation analysis revealed that calculated *C. forsteri* ITS2 rDNA copy counts per mg of heart was not correlated with adult fluke numbers in the heart flush of the same individual SBT (R^2=0.0407, P=0.1254).

2.4. Discussion

The qPCR assay developed and validated in this study represents the first quantitative hydrolysis probe-based assay for *C. forsteri*, *C. orientalis* and *C. opisthorchis* detection. While qPCR assays for the detection of *C. forsteri* and *C. orientalis* have been developed, they have relied on the use of DNA intercalating SYBR green chemistry which although sensitive and specific required expert interpretation of melt curve analysis at *Cardicola* DNA concentrations approaching the assays LOQ and LOD (Polinski et al., 2013a). In contrast the qPCR assay and species specific primers presented here were able to reliably provide unambiguous and rapid absolute quantification of all three species from the genus *Cardicola* known to infect bluefin tuna, while remaining both exquisitely sensitive and specific at low target analyte concentrations.

This improved qPCR assay was used to identify and quantify *C. forsteri* and *C. orientalis* ITS2 rDNA in wild and ranched SBT heart and gill samples. This molecular method was superior in terms of speed, sensitivity and specificity compared to traditional methods of *Cardicola* detection and diagnosis such as heart flushes and histology that are both time consuming and labor intensive (Kirchhoff et al., 2011b, Aiken et al., 2006, Shirakashi et al., 2013). In contrast the qPCR assay described here can be performed in as little as 2 h starting from gill or heart samples providing rapid detection, species identification and quantification of pathogen load measured in terms of DNA quantity. Furthermore, the simplicity of the qPCR provides an opportunity for this assay to be adapted to a point of care assay that could be used at the site of sampling or on a nearby vessel.

Prior to 2010, *C. forsteri* was believed to be the only species of *Cardicola* infecting SBT. The mean prevalence of infection with *C. forsteri* was 62.64% using heart flushes (Aiken et al., 2015). Methods of detection and species differentiation have since become more sensitive and

precise, where microscopy methods have led to the description of *C. orientalis* in PBT in 2010 (Ogawa et al., 2010) and qPCR methods consequently to the confirmation of the species in SBT (Shirakashi et al., 2013, Polinski et al., 2013a). *C. orientalis* has since been identified as the predominant species of *Cardicola* having been found in 86% of SBT samples from 2008-2012 whilst *C. forsteri* was present in 36% of SBT, that were examined using qPCR (Polinski et al., 2013a). Hence, the absence of *C. orientalis* in 2014 and 2015 was unexpected, as this species had been documented as the main blood fluke species in ranched SBT populations in the previous five years (Polinski et al., 2013a).

Using qPCR methods, future studies should determine the dynamics of prevalence of different species of *Cardicola* throughout the season, particularly before and after PZQ treatments. In 2013 PZQ was introduced in the Australian SBT industry as a method of mitigating infections with blood fluke and has been applied since by the industry. The change of the most prevalent species of *Cardicola* may demonstrate differences in anthelmintic efficacy against the two blood fluke species.

Traditional methods including heart flush data from previous studies reported a 5-10% prevalence of *C. forsteri* in wild juveniles caught in the Great Australian Bight in 2001 (Colquitt et al., 2001c, Aiken et al., 2006, Kirchhoff et al., 2012, Kirchhoff et al., 2014). Comparatively low prevalence of *Cardicola* spp. prevalence in wild SBT (compared to ranched) indicates that levels of infection seen in ranched systems could result from increased prevalence of the infected intermediate host in the ranching areas, thereby facilitating completion of the parasite's life-cycle (Cribb et al., 2011, Cribb et al., 2000). To date, all three species of *Cardicola* known to infect tuna have been detected in ranched PBT and ABT (Forte-Gil et al., 2016, Shirakashi et al., 2016b, Ybañez et al., 2011).

The proximity of ranched SBT to *C. forsteri* and *C. orientalis* intermediate hosts could propagate the infection of ranched SBT (Montero et al., 1999, Bullard and Overstreet, 2002b, Bullard and Overstreet, 2002a, Munday et al., 2003). Confined SBT probably come in contact with larger numbers of cercariae than wild, free swimming SBT, thereby increasing the likelihood of infection (Bullard and Overstreet, 2002b). In a previous study, off-shore relocation of sea cages resulted in no *C. forsteri* and *C. orientalis* infection and maximized fish growth (Kirchhoff et al., 2011b).

In wild PBT juveniles annual prevalence of *C. orientalis and C. opisthorchis* was highly variable (Sugihara et al., 2016, Pennacchi et al., 2016). Quantitative PCR showed that *C. orientalis* prevalence was 20.9% in 2011 and dropped below 5% the following years. *C. opisthorchis* prevalence in wild PBT ranged between 74.6% in 2011, 4.1% in 2013, and 32.1% in 2014 (Sugihara et al., 2016). In ABT no statistically significant difference in prevalence was detected among the three detected blood fluke species in either wild and ranched specimen (Forte-Gil et al., 2016). Prevalence of blood flukes in ABT was 6.5% and 37.5% when only a single blood fluke species was detected, whereas it was 60.9% and 12.5% for prevalence of all three species in wild and cultured respectively (Forte-Gil et al., 2016). Forte-Gil et al. (2016) suggested that the intermediate hosts of *C. forsteri*, *C. orientalis* and *C. opisthorchis* may be found along the Mediterranean coast.

Sampling location, particularly in the gill, may affect the documented prevalence of each species of *Cardicola*. Adult *C. forsteri* reside primarily in the heart, while adult *C. orientalis* have been found in the afferent gill artery, branchial arteries as well as in the heart (Shirakashi et al., 2012b, Ogawa et al., 2011, Ogawa et al., 2010). In the present study, the central filaments of the left second gill arch were analyzed to assess *Cardicola* infection. This area has been shown to give

most accurate indication of species composition and prevalence (Santos et al., 2012, Shirakashi et al., 2012b). Therefore, when investigating *C. forsteri* and *C. orientalis* prevalence in gill the inclusion of the afferent artery in analyses may contribute to a clearer exposition of species prevalence.

2.5. Conclusion

This hydrolysis probe-based qPCR assay will contribute to improved understanding and quantification of blood flukes from genus *Cardicola* prevalence in ranched and wild SBT. In addition, this study highlights the first detection of *C. forsteri* and *C. orientalis* in wild SBT caught along the Tasmanian coast (Pedra Branca), this being the first documentation of the latter in wild SBT.

3. Detection of *Cardicola forsteri* and *Cardicola orientalis* in sea water

3.1. Introduction

In environmental research, methods such as PCR and qPCR are often used to assess presence of bacteria, viruses, and protozoans in water (Shannon et al., 2007, Hyman and Collins, 2012). In some cases molecular methods are the only way to detect pathogens, as target analyte levels may be too low for culture methods, if culturable at all, while still high enough for infection. Though molecular methods do not provide information regarding the infectivity of the target pathogen, qPCR is a valuable risk assessment tool presenting rapid and species specific quantitative information regarding the target segment of the waterborne pathogens genome, be it RNA or DNA (Girones et al., 2010). This species specificity has given nucleic acid-based methods a unique advantage over more traditional methods such as microscopy, immunological and/or flow cytometric methods.

More specifically, fluorescent hydrolysis probe-based qPCR methods have been confirmed as a rapid, cost-effective tool to provide information regarding the presence, quantity, and distribution of pathogens in water (Shannon et al., 2007, Lee et al., 2006, Lee et al., 2008, Girones et al., 2010). This tool has proven to be particularly valuable for the detection of marine and aquaculture parasites during their free-living life cycle stages. For example, the development of one such qPCR assay for detection of parasites belonging to the *Hemotodinium* spp. during their waterborne free-living life cycle stage provided key information regarding duration of their infection of blue crabs in estuarine habitats (Li et al., 2010). Similarly, in China a hydrolysis probe-based qPCR assay has been used to assess the cercarial presence of *Schistosoma japonicum* in rivers, a parasite known to put more than 30 million people at risk in endemic areas (Hung and Remais, 2008). In addition, this method has been successfully applied to the rapid

detection and quantification of *Neoparamoeba perurans*, the causative agent of amoebic gill disease (AGD) in salmon, in the marine environment (Bridle et al., 2010).

Cercariae and miracidia, the infectious life stages of blood flukes, are present in water for short periods of time and their rDNA may be quantifiable using molecular methods (Cribb et al., 2011). Quantitative PCR analysis of SBT organs, specifically heart and gill, has proven to be more sensitive and precise at identifying the presence of *C. forsteri* and *C. orientalis* than previously used methods, such as histology and heart flush microscopy (Polinski et al., 2013b) (see Chapter 2). The sensitivity, verified accuracy and applicability of qPCR for *Cardicola* spp. has made it the most promising of detection methods, though it is unable to distinguish between life cycle stages of the blood fluke (Polinski et al., 2013a, Polinski et al., 2013b). In addition, a non-lethal method assessing *Cardicola* spp. infection in SBT would be beneficial given the high market value of each specimen.

The detection of blood fluke DNA from water surrounding ranched SBT could be a useful tool for determining risk of infection with *Cardicola* spp. In this study, the presence of *C. forsteri* and *C. orientalis* ITS2 rDNA was examined in water from sea cages containing SBT compared to control locations 3.5 km North and 2 km South of sea cages. The use of quantification of *C. forsteri* and *C. orientalis* DNA in water as a risk assessment and the potential for a non-invasive method of *C. forsteri* and *C. orientalis* diagnosis early in the SBT ranching season was assessed.

3.2. Materials and Methods

3.2.1. Field collection and processing of samples

Water samples were collected between July and November 2012, and July 2013 and July 2014 from locations in the Boston Bay area and inside SBT ranching cages, which were being harvested at that time (Figure 6). Control sampling locations were 3.5 km (North) and 2 km (South) away from tuna sea cages (Figure 6). Sample volume and depth of collection are shown in Table 5. Boston Bay depth map illustrated in Figure 7.

In 2013, to investigate the implication of volume, sample replicate quantities were altered from 3 x 1 L per location to 2 L, 1 L and 500 mL. All 2014 samples were 1.5 L with 6 replicates from the surface and 6 from 10 m depth. For each sample, divers filled a sterile bottle with seawater at the chosen depth (sometimes this took place during/after fish harvest). Within 7 hours of collection the water was filtered through a 47 mm diameter GF/C Whatman™ 1.2 μm glass microfiber filter, in a Nalgene™ (Thermoscientific) reusable filter holder (Wright et al., 2015). Using forceps, the filter was removed and preserved in 1 mL lysis buffer (4 M Urea, 1% SDS, 0.2 M NaCl, 1mM Na Citrate) in 5 mL vials, kept at room temperature (RT) approximately 21°C for 10 min to ensure that the filter was completely saturated with lysis buffer and then stored at -80°C for qPCR analysis.

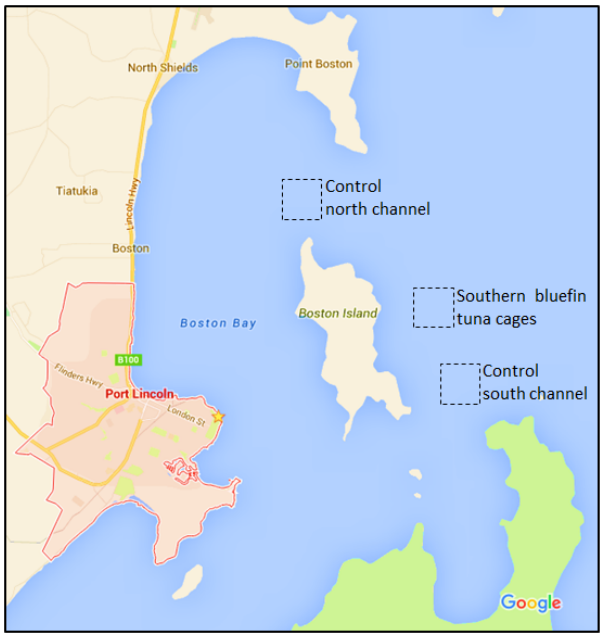

Figure 6 Water sampling locations (Maps, 2016)

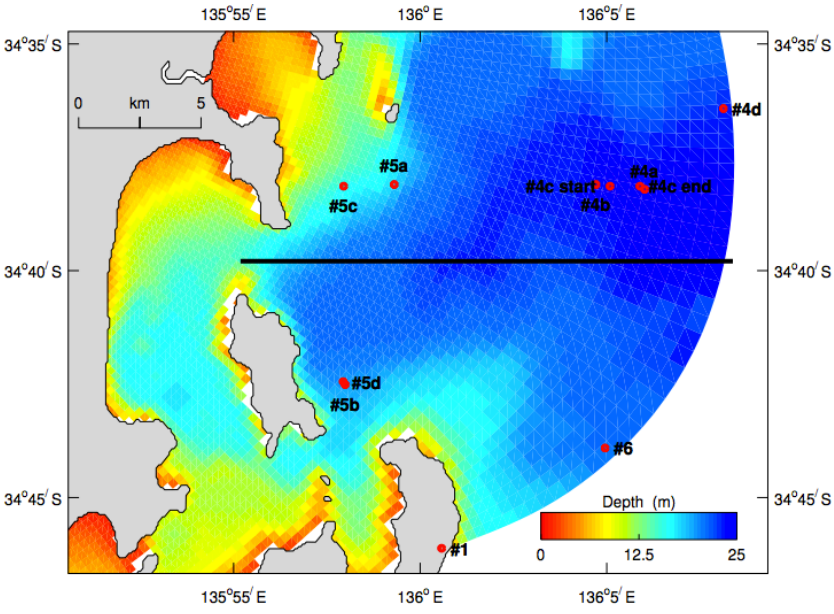

Figure 7 Boston Bay depth map (Herzfeld et al., 2008)

Table 5 Water collection over three years inside active SBT sea cages and control locations

Sampling date[1]	Location[3]	Depth (m)	Volume (L)	Total samples	SBT present Yes/No
11.07.12 harvest	Cage A	0	1	6	Y
12.07.12 harvest	Cage B	0	1	3	Y
		5	1	3	Y
13.07.12 harvest	Cage C	0	1	6	Y
		5	0.85, 0.9, 0.75	3	Y
29.08.12 harvest	Cage C	1	1	2	Y
	Cage A	1	1	2	Y
	Cage B	1	1	2	Y
30.08.12 harvest	Control South[4]	n/a[2]	1	3	N
	Control North[4]	n/a[2]	1, 0.95, 0.9	3	N
14.11.12 Non-harvest, no SBT on farms	Control North[4]	0-1	0.9, 1, 1	2	N
	Control South[4]	0-1	1, 1, 0.9	2	N
	Cage A	0-1	1	3	Y
	Cage B	0-1	1	3	Y
21.06.13 harvest	Control North[4]	0-1	2, 1, 0.5	3	N
	Cage D	0-1	2, 1, 0.5	3	Y
	Control South[4]	0-1	2, 1, 0.5	3	N
12.07.14 harvest	Cage E	0	1.5	6	Y
		10	1.5	6	N

[1]Some of the 2012 1 L samples ranged between 0.75 L and 1 L. This can be attributed to 1 L sampling bottle not being filled completely due to adverse sampling conditions

[2]Samples not available.

[3]Where Cages A-E, Control N and S were located at locations illustrated in Figure 6.

[4] Control sampling locations were 3.5 km (North) and 2 km (South) away from tuna sea cages.

3.2.2. Nucleic acid extraction

Each sample containing the filter and 1 mL lysis buffer was incubated at 55°C for 30 min after the addition of 5 µL proteinase K (Bioline). Total nucleic acid (TNA) was removed from the filter by centrifugation at 16,000 x g for 5 min at 18 °C. Remaining nucleic acid extraction protocol was conducted as described in Chapter 2.

3.2.3. Real-time qPCR *C. forsteri* and *C. orientalis* detection

Samples collected in 2012 were examined using an assay exclusively using SYBR® Green 1 chemistry, as described by (Polinski et al., 2013a). Samples collected in 2013 and 2014 were examined using the hydrolysis probe-based assay described in Chapter 2. Due to the relative ease of use while maintaining equivalent sensitivity and specificity the hydrolysis probe-based assay was adopted as the primary method of *C. forsteri* or *C. orientalis* ITS2 rDNA detection for this study after validation in 2013. Water samples collected and analyzed in 2012 could not be retroactively screened because the entire sample was used during the 2012 examination (as with the analysis conducted in the following years). Once the DNA is extracted from the filter (as described in this chapter) the DNA is quantified and remaining dilutions are disposed of if not used within 48 hours due to DNA degradation. To confirm that assay inhibition was not present, low amounts of DNA were spiked into extractions that had previously given negative results and upon reanalysis gave positive results. This removed any question as to whether PCR inhibitors were preventing amplification. Combined with dilutions and efficiency measurements gives very high confidence in the assay. In addition, examinations where no *C. forsteri* or *C. orientalis* ITS2 rDNA was detected were repeated using a 1:2 or 1:5 dilution of the original pellet re-suspension was used to confirm the result.

3.2.4. Statistical analysis

GraphPad Prism 5 (GraphPad software, CA, USA) was used to run the one-way analysis of variance of qPCR detection of *C. forsteri* and *C. orientalis* to assess significance of sampling location. P values less than 0.05 were considered significant. Shapiro-Wilk's test was used to test the assumption of normality. Homogeneity of variance was assessed using Levene's test of homogeneity of variances. Data is presented as mean ± 95% Cl. An independent-samples t-test was run to determine if there was a difference in the prevalence of *C. forsteri* and *C. orientalis*.

3.3. Results

Quantitative PCR analysis to detect *C. forsteri* and *C. orientalis* in water collected during SBT harvest in three consecutive years indicated the presence of blood fluke DNA in samples (n = 10) collected on 29th and 30th of August, 2012 only.

A one-way ANOVA was conducted on data from samples collected on 29th and 30th of August, 2012, to determine if the sampling location affected the prevalence of *C. forsteri* and *C. orientalis* ITS2 rDNA detected. There were no outliers, as assessed by boxplot; data was normally distributed for each location, as assessed by Shapiro-Wilk test, and there was homogeneity of variances, as assessed by Levene's test of homogeneity of variances. Tukey's post hoc analysis revealed that Control North was significantly different from the other locations,

both *C. forsteri* and *C. orientalis* ITS2 rDNA were statistically significantly less prevalent at this location (Figure 8).

The mean *C. forsteri* ITS2 rDNA copies number per liter in cage water samples was 230.67, while the mean for the control north and south locations were 0 and 769.33 respectively. The mean *C. orientalis* DNA quantified from cage water was 4480.83 copies, and 215 and 10363.66 copies per liter in North and South controls, respectively.

An independent-samples t-test was run on data from samples collected on 29th and 30th of August, 2012, to determine if there was a difference in the prevalence of *C. forsteri* and *C. orientalis* at each location. There were no outliers in the data, as assessed by inspection of a boxplot. Detected ITS2 rDNA copy numbers were normally distributed, as assessed by Shapiro-Wilk's test, and there was homogeneity of variances, as assessed by Levene's test for equality of variances. Significantly more *C. orientalis* ITS2 rDNA (M = 4885.08, SD = 7328.71) was detected compared to *C. forsteri* (M = 232.67, SD = 337.82) in samples collected on 29th and 30th of August, 2012.

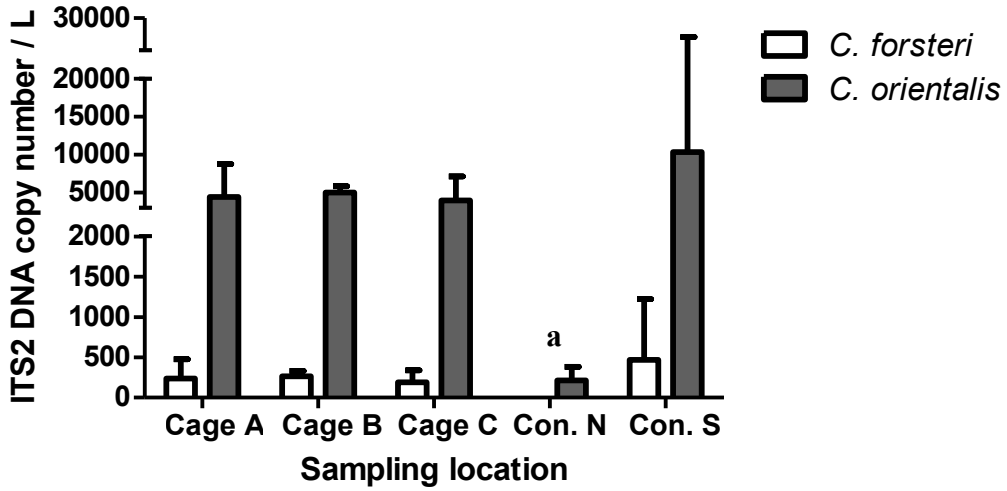

Figure 8 *Cardicola forsteri* and *Cardicola orientalis* ITS2 DNA copy numbers detected in independent duplicate 1 L water samples from 1 m depth from five locations in Boston Bay, Port Lincoln, SA, 2012. Cage water samples were collected on the 29th August (2012) and control locations on the 30th August (2012). Control North and South were 3.5 km and 2 km away from SBT sea cages, respectively. Data shown are the mean, the bars indicate 95 % Cl. Post hoc analysis showed significantly fewer *Cardicola forsteri* and *Cardicola orientalis* ITS2 rDNA copies were detected in samples collected the Control North (a) compared to Cage and Control South locations ($P < 0.05$). T-test analysis showed that significantly more *C. orientalis* ITS2 rDNA copies were detected compared to *Cardicola forsteri* ITS2 rDNA in both cage and control samples ($P < 0.05$).

No *C. forsteri* and *C. orientalis* ITS2 rDNA was detected in July 2013 and 2014 water samples collected at control sites or live tuna sea cages. No *C. forsteri* and *C. orientalis* DNA was detected in any of the replicates (2 L, 1 L, and 0.5 L) in 2013 samples (n = 9). In addition, no *C. forsteri* and *C. orientalis* DNA was detected at either of the sampling depths (0 m and 10 m, n = 12).

3.4. Discussion

This study has successfully applied a qPCR assay to detect *C. forsteri* and *C. orientalis* ITS2 rDNA from water samples collected in SBT sea cages and two control locations. Though only detected in the first of three sampling years, the species composition documented in August 2012 water samples was similar to the prevalence seen in qPCR analysis of heart and gill from SBT kept at the same location the same season (Polinski et al., 2013a, Polinski et al., 2013b). In heart and gill samples *C. orientalis* was prevalent in 84% and 62% of SBT, whereas *C. forsteri* was only found in 5% and 2% respectively (Polinski et al., 2013a). In addition, overall prevalence of

each *Cardicola* species per examined organism was 86% (*C. orientalis*) and 36% (*C. forsteri*) (Polinski et al., 2013a). Therefore, when detected, qPCR of water may be indicative of *C. forsteri* and *C. orientalis* infection ratios in SBT.

The 2013 introduction of the antithelmintic PZQ as a treatment for *C. forsteri* and *C. orientalis* infection in Australian ranched tuna resulted in a reduction of reported annual mortalities, from 10-15% in the previous years to less than 1% since (Polinski et al., 2014). The reduction of *Cardicola* species infection in ranched SBT, specifically the massive reduction of *C. orientalis* prevalence may have contributed to the lack of *C. forsteri* and *C. orientalis* detection in the present study. One could speculate that with the improved management of *Cardicola* species induced infection and mortalities in ranched SBT the Boston bay area saw a reduction in *C. forsteri* and *C. orientalis* prevalence, and with that no detection in 2013 and 2014.

The lifespan of the infectious free-living life cycle stages of parasites are relatively short and highly variable depending on ambient light and water temperature (Lo and Lee, 1996, Olsen, 1986). For example, the lifespan of cercariae of the trematode parasite known to infect black carp; *C. formosanus* was 160 h at 15°C, whereas in suboptimal conditions the lifespan can be diminished to 30 h (Lo and Lee, 1996). Similarly, the average lifespan of *C. formosanus* miracidia is 20 h (Ashton et al., 2001). Given the relatively short lifespan of infectious free-living life cycle stages of parasites, compared to adults which have been reported to life up to 5 years (Olsen, 1986), the water 2013 and 2014 samples utilized in the present study may have been collected in a time where no *C. forsteri* or *C. orientalis* miracidia or cercariae were present in the water.

Both *C. orientalis* and *C. forsteri* were significantly less prevalent in the control North location in comparison to cage and control South 2012 samples. The control North sampling location was in shallower (≈ 15 m) compared to cage and control South (≈ 20 m) (Herzfeld et al., 2008). A previous study showed that moving sea cages further offshore (deeper water) resulted in lower *Cardicola* species infection levels in SBT in the respective cages (Kirchhoff et al., 2011b). Given the results of this study, one may speculate that parameters other than depth, such as water salinity or temperature may affect the prevalence of Cardicola species ITS2 rDNA in seawater.

Currently, water qPCR data cannot be used as an infection assessment tool for *C. forsteri* and *C. orientalis* in SBT but rather should be interpreted in close correspondence with qPCR analysis of SBT organs or heart flush microscopy adult fluke counts. A similar study working to detect and quantify *Ceratomyxa shasta* in river samples found no correlation between spore numbers and mortality in exposed groups (Hallett and Bartholomew, 2006), indicating that water based parasite DNA quantification on its own may not be sufficient information to accurately estimate infection. The method quantifying *C. forsteri* and *C. orientalis* ITS2 rDNA from sea water demonstrated in this study requires further development and validation before being useful for surveillance, risk assessment and intervention planning during ranching operations. In addition, future studies could continue to explore the prevalence of *C. forsteri* and *C. orientalis* cercariae/miracidia in the water column. A study using qPCR for the detection of *Schistosoma japonicum* in water suggested the use of high volume filtration methods to target the respective cercariae's surface seeking nature in low parasite density conditions (Haas et al., 1987, Hung and Remais, 2008). A better understanding of spatial distribution in water column of infective *C. forsteri* and *C. orientalis* life stages and examination of high volume filtration methods may improve reliability of water quantification results in relation to SBT infection levels.

The analysis of environmental DNA has recently become an increasingly valuable resource, particularly in ecosystem management or farming conditions where the identification of a target species is the goal (Thomsen et al., 2012). Further, the rapid detection and specificity provided by molecular methods makes analysis of environmental DNA suitable for a point of care systems where on-site results are needed (Wang et al., 2016). Future studies exploring the use of POC systems for the detection of *Cardicola* spp. ITS2 rDNA in sea water may choose to examine alternative PCR methods such as recombinase polymerase amplification (Piepenburg et al., 2006) or loop-mediated isothermal amplification (Notomi et al., 2000), which may provide faster results while limiting specialized laboratory equipment needed (Wang et al., 2016, Gubala et al., 2011). In addition, the goal of faster on-site DNA detection could be explored with novel methods such as laser transmission spectroscopy (Mahon et al., 2012) or the application of environmental DNA analysis to carbon nanotube platforms (Mahon et al., 2011). Some studies have combined the analysis of environmental DNA with next generation sequencing, which is able to detect multiple species simultaneously and poses to be a valuable tool for monitoring species diversity in bodies of water (Thomsen et al., 2012).

The wide range of methodologies published for the detection of target DNA from environmental samples (fresh and sea water) currently inhibits the coining of a single standard method (Rees et al., 2014). For example, the volume of water sampled is still largely dependent on the source, whereby significantly larger samples are required when sampling moving water in comparison to standing water. Further, the full scope of how environmental conditions affect the degradation and detection of DNA in water is yet to be fully understood.

This study presents the prevalence *C. orientalis* and *C. forsteri* in Boston Bay, Port Lincoln, Australia. *C. forsteri* and *C. orientalis* DNA was only detected in the first (August 2012) of three

consecutive SBT sampling years, despite the presence of at least one blood fluke species in SBT harvested in 2013 and 2014 at the time of water sampling (see Chapter 2). Quantitative PCR analysis of water should not be used as the sole method of detection and justification for treatment of *C. forsteri* and *C. orientalis* in ranched SBT. Future studies should investigate the detection and quantification of *Cardicola* spp. from seawater throughout the ranching season.

4. Comparison of DNA extraction methods from formalin-fixed paraffin-embedded organs of Southern bluefin tuna (*Thunnus maccoyii*) to determine the presence of *Cardicola forsteri* and *Cardicola orientalis*

4.1. Introduction

Molecular analysis can be conducted on highly degraded DNA, such as insects embedded in amber or dried blood (Shibata, 1994). The purpose of formalin-fixed paraffin-embedding (FFPE) is to preserve three-dimensional cellular structure of a sample (Shibata, 1994, Gilbert et al., 2007). With molecular methods becoming more established for pathogen detection, sample fixation is increasingly important; to allow for both histological and molecular analysis from the same sample. Samples fixed in 10% neutral buffered formalin are suitable for DNA extraction, conversely buffered formal sublimate (B-5) fixation has rendered poor DNA extraction efficiency (Shibata, 1994). In a study investigating the detection of *Neoparamoeba perurans* in salmon gills, seawater Davidson's and PAX-gene® provided good DNA extraction efficacy and quantity for qPCR analysis, while maintaining organ morphology for histological examination (Cadoret et al., 2013).

Traditional methods of detection of blood flukes from genus *Cardicola*, the main health concern in ranched SBT (Colquitt et al., 2001b, Cribb, 2000, Munday et al., 2003), have relied heavily on microscopic observation of heart flushes or histological analysis as a primary means of diagnosis. The emergence of qPCR has provided a rapid and highly sensitive method of detecting pathogens and more recently has been applied to the *Cardicola* genus as a whole (Polinski et al., 2013b, VanGuilder et al., 2008). The advent of hydrolysis probe-based real time quantitative PCR and cross referencing of nucleic acid databases further improves accuracy in pathogen detection. Coinciding with the formal description of *C. orientalis* in 2010 (Ogawa et al., 2010), Polinski et al. (2013) re-examined archival 2008 serum and 2010 FFPE heart and gill

SBT samples using qPCR (Polinski et al., 2013a, Belworthy, 2012). Quantitative PCR was successfully used to identify presence of *C. orientalis* in archival SBT serum samples from 2008.

The limited supply of archival FFPE samples calls for a highly efficient DNA extraction method guaranteeing maximum DNA extraction for qPCR analysis. Previous DNA extraction of SBT heart from FFPE samples consisted of two 10 minute incubations with xylene followed by two 10 minute washes with 100% alcohol (Polinski et al., 2013a). The use of QIAamp DNA mini kit® (Qiagen) after proteinase K digestion, showed improvements to DNA extraction efficacy from FFPE samples (Farrugia et al., 2010).

The aim of this study was to examine the efficacy of 5 DNA extraction methods and subsequent detection of *C. forsteri* and *C. orientalis* from FFPE SBT organs. The impact of section thickness, influence of xylene deparaffinization, and incubation time was explored. The most effective method was applied to archival FFPE SBT samples to determine the presence of *C. orientalis* preceding documented presence in 2008 SBT.

4.2. Materials and Methods

4.2.1. Archival samples

Archival histological samples of SBT heart and gill from Port Lincoln from 1995 (n = 10) and 2004 (n = 10) were obtained from the archival collection at University of Tasmania. Sampling time was within the 6-month ranching period but the exact dates were unknown. Histology sections from each archival paraffin embedded sample were stained with haematoxylin and eosin to confirm presence of *Cardicola* spp. eggs.

4.2.2. 2014 ranched SBT samples

2014 SBT (n = 10) heart and gill samples were collected during commercial harvest approximately 20 weeks after being transferred to sea cages. Heart and gill samples for histology were collected and kept at 4 °C until processing later that day. For a more detailed sampling methodology please refer to Chapter 2, section 2.2.3.

After 24 h fixation in 10 % neutral buffered formalin samples were transferred to 70 % ethanol for storage. Gill samples were decalcified in Rapid decalcifying fluid (Australian Bio-stain Pty. Ltd.) for 2 h prior to being dehydrated using a sequence of alcohols at increasing concentrations (80 %, 95 % and 100 %), embedded in paraffin, sectioned at 5 µm and then stained with Haematoxylin and Eosin (H&E) using standard methods (Brown, 2002).

4.2.3. Nucleic acid extraction from fixed paraffin-embedded tissues

4.2.3.1. Comparison of 5 DNA extraction methods

Five FFPE DNA extraction methods were examined in this study (Table 6). This consisted of with (method 1) and without (method 2) xylene deparaffinization as described previously in FFPE SBT DNA extractions (Polinski et al., 2013a), without xylene deparaffinization but including a 1 h 90 °C incubation post proteinase K incubation (method 3), a commercial FFPE DNA extraction kit (QIAamp DNA FFPE Tissue Kit) (method 4), and a TRIzol reagent-based method (method 5). Each method consisted of ten independent 10 µm SBT heart sections. The

blocks used were known positive samples, which confirmed via qPCR analysis of RNA preservation reagent preserved samples from the same organ.

Method 1-3 samples were suspended in 500 µL extraction buffer (4 M Urea, 0.5 % SDS, 0.2 M NaCl, 10 % glycerol) containing 5 µL proteinase K (Bioline) and incubated at 37 °C overnight. Method 3 samples were incubated at 90°C for 1 h while method 1 and 2 samples remained at room temperature (21°C) (RT) (Table 6). Samples were then cooled on ice for 5 min; and protein was precipitated by adding 350 µL 7.5 M ammonium acetate, 20 s vortex and centrifugation at 14,000 x g for 3 min at 18°C. DNA was precipitated from the supernatant by adding equal volume of isopropanol followed by centrifugation at 16,000 x g for 30 min. The resulting DNA pellet was rinsed twice with 1 mL 75 % ethanol and re-suspended in 35 µL molecular water (0.1 % Triton X-100 Sigma-Aldrich®, 10 mM Tris).

The commercial kit (method 4) used in this study was the QIAamp DNA FFPE Tissue Kit (QIAGEN). The kit was used as per manufacturer instructions.

For TRIzol-based (method 5) DNA extraction from FFPE SBT heart paraffin was removed using xylene deparaffinization, samples were suspended in 500 µL extraction buffer (4 M Urea, 0.5 % SDS, 0.2 M NaCl, 10 % glycerol) containing 5 µL Proteinase K (Bioline) and incubated at 60 °C for 60 min. The solution was homogenized with the addition of 1 mL TRIzol and a power homogenizer (IKA®). The ten samples were incubated at RT for 5 min, followed by the addition of 50 µl 4-bromoanisole (MRC, Inc). After 3 min incubation at RT, sample was centrifuged at 16,000 x g for 15 min at 4 °C. The resulting aqueous phase was removed, placed into a new 1.7 mL tube and 300 µL isopropanol added. Following 3 min RT incubation sample was centrifuged

at 2000 x g for 5 min at 4 °C. The resulting DNA pellet was rinsed twice with 1 mL 75 % ethanol and re-suspended in 35 µL molecular water (0.1 % Triton X-100 Sigma-Aldrich®).

Table 6 Presence/absence of xylene deparaffinization, proteinase K incubation and 90°C 1 h incubation steps methods 1-5

Method #	Xylene deparaffinization (Y/N)	Proteinase K incubation (Y/N)	90°C 1 h incubation (Y/N)
1	Y	Y	N
2	N	Y	N
3	Y	Y	Y
4	N	N	Y
5	Y	Y	N

Illustration of the 5 DNA extraction methods used in this study and the presence/absence of a xylene deparaffinization, proteinase K incubation and 90°C 1 h incubation step in each respective method. "Y" denotes the presence of this step, and "N" denotes the absence of this step in the method protocol.

4.2.4. Consecutive FFPE sections

Ten consecutive 10 µm sections from positively identified (qPCR analysis of samples preserved in RNA preservation reagent from the same respective SBT) (see chapter 2) heart and gill paraffin blocks from July 2014 SBT harvest were individually analysed using qPCR to investigate possible *C. forsteri* and *C. orientalis* DNA presence variation within sections of each organ. DNA extraction was conducted using method 3.

4.2.5. Archival 1995 and 2004 FFPE heart

Ten 10 µm thick sections (total of 100 µm per tube) from 1995 (n = 10) and 2004 (n = 10) FFPE SBT heart blocks were analysed for *C. orientalis* DNA. If positive, extraction and qPCR was repeated on new sections 2 times to confirm the result. DNA extraction included 1 h 90 °C incubation after proteinase K incubation.

4.2.6. Section thickness and incubation time

For the examination of the effect of section thickness and incubation time on DNA extraction efficacy duplicate 1.7 mL tubes containing either 2 x 5 µm heart sections (n = 6) or 1.7 mL tubes containing 1 x 10 µm heart sections (n = 6) were incubated for 24 h (12 tubes) overnight (ON) and 72 h (12 tubes) at 37 °C. DNA extraction was conducted using method 3.

4.3. Real-time PCR *C. forsteri* and *C. orientalis* detection

Please refer to Chapter 2 for *C. forsteri* and *C. orientalis* primer/probe sequences. Specificity of F and R primers was shown by Polinski et al (Polinski et al., 2013a). A CFX Connect Real-time PCR Detection System (Bio-Rad, NSW, Australia) was used for real-time qPCR analyses. For *C. forsteri* and *C. orientalis* detection, 2X MyTaq™ HS mix was used in combination with 150 nM species-specific probe; 400 nM forward and reverse primer, and molecular water. Probes were labeled at the 5' end with 6-carboxy-2,4,4,5,7,7 -hexachlorofluorescein succinimidyl ester (HEX) together with a Black Hole Quencher® (BHQ, Biosearch Technologies, CA, USA) added to the 3' terminus. Cycling conditions comprised an activation of DNA polymerase at 95°C for 3 min, followed by 45 cycles of 10 s at 95°C and 30 s at 60 °C, with relative fluorescence

measured at the end of each 60 °C extension. Quality of extracted DNA was not evaluated in the present study because the primary aim was to examine the efficacy of 5 DNA extraction methods (using the same FFPE blocks) and subsequent detection of *C. forsteri* and *C. orientalis,* rather than elucidating fixation induced DNA degradation as previously documented by Cadoret et al. (2013).

4.4. Statistical analysis

GraphPad Prism 5 (GraphPad software, CA, USA) was used to run one-way analysis of variance (ANOVA) of qPCR detection of *C. forsteri* and *C. orientalis* in SBT heart and gill. Two way ANOVA was used to determine significance of section thickness and incubation time on DNA extraction of FFPE SBT heart. Highly variable data were log + 1 transformed to adequately present scale. Difference among groups was confirmed using Tukey's post hoc. $P < 0.05$ was considered significant. Shapiro-Wilk's W test was used to test assumption of normality. Levene's F test was used to confirm homogeneity of variances.

4.5. Results

The method 3 (sample incubation for 1 h at 90 °C post proteinase K incubation) resulted in a 100-fold increase in DNA yield compared to without (methods 1 and 2) and an approximate 10,000-fold increase compared to method 5 that incorporated a traditional organic extraction protocol (Table 6). While method 3 DNA yield was also significantly higher compared to method 4 which used a Qiagen QIAamp FFPE kit, method 4 DNA yield was significantly higher than methods 1, 2, and 5. Xylene deparaffinization prior to proteinase K incubation made no

significant difference in final yield of calculated *C. forsteri* ITS2 rDNA copies and no differences in yield were seen among methods 1, 2 or 5. The total target analyte ITS2 rDNA detected from single 10 µm FFPE sections varied considerably across extraction methods (Figure 9).

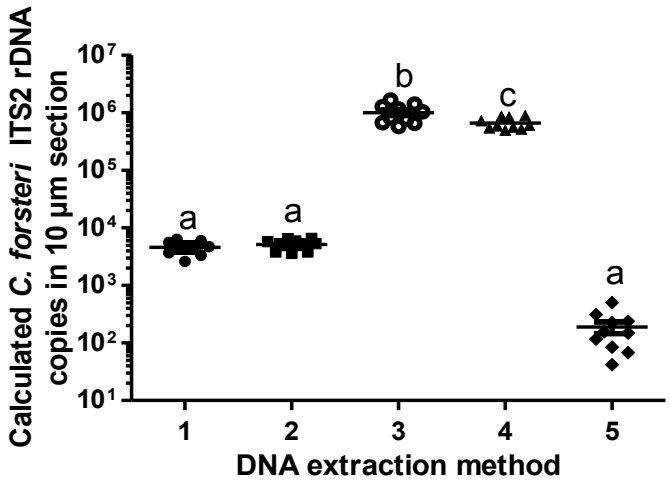

Figure 9 Comparison of quantified *Cardicola forsteri* DNA extraction from 10 x 10 µm sections of the same SBT heart block. The methods were with (method 1) and without (method 2) 2 x 10 min xylene deparaffinization steps prior to proteinase K overnight incubation, standard non-organic DNA extraction including 1 h at 90°C post proteinase K incubation (method 3), the Qiagen QIAmp FFPE DNA Tissue kit (method 4), and a traditional organic extraction protocol (method 5). Line represents the mean. Different letters denote significant differences at $p < 0.05$.

C. forsteri and *C. orientalis* DNA copy numbers within the FFPE organ based on consecutive section analysis were highly variable, with a mean coefficient of variance of 78.95% in heart (Figure 10A) and 58.87% in the gill (Figure 10B). *C. orientalis* was not found within the FFPE heart and gill samples from 2014, however this species was detected using qPCR analysis of

samples collected from gills and heart of the same individual and preserved in RNA preservation reagent sample (see Chapter 2).

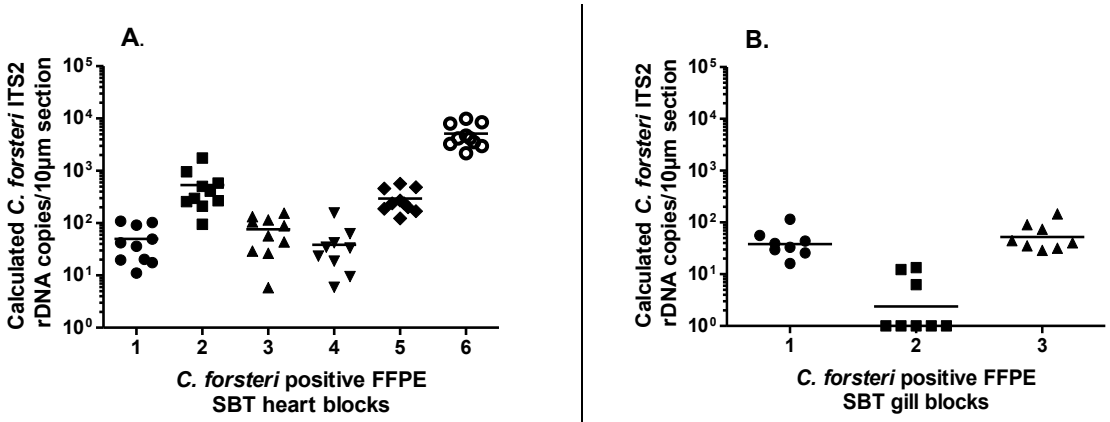

Figure 10 *Cardicola forsteri* ITS2 rDNA copy numbers in 10 x 10µm sections in 6 SBT heart (A) and 3 gill (B) FFPE blocks. Heart FFPE independent consecutive section qPCR analysis showed a mean 73.9% coefficient of variance. Independently analyzed consecutive FFPE gill sections had a mean coefficient of variance of 58.87%. The line represents the mean.

There was no significant interaction between section thickness and incubation time on the final copy numbers of *C. forsteri* DNA in FFPE SBT heart (Figure 11). Extended incubation at 37 °C for 72 h instead of 24 h, resulted in a significant increase in *C. forsteri* ITS2 rDNA (Figure 11). No significant difference in ITS2 rDNA copy numbers was seen between sections of different thickness.

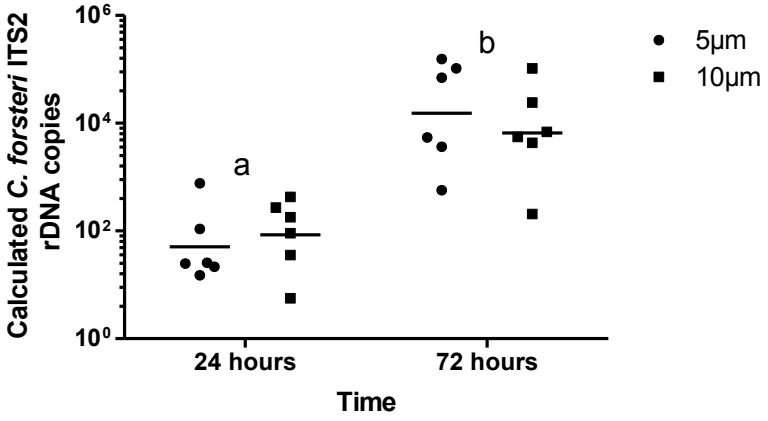

Figure 11 Extraction efficiency based on section thickness (2 x 5µm sections vs. 1 x 10µm) and proteinase K incubation (for 24 h and 72 h at 37 °C) time. Data were transformed (log + 1) for scale and line represents the mean. Different letters denote significant differences between the 3 incubation times at $p < 0.05$.

Molecular analysis of 20 archival FFPE blocks identified a single 1995 heart sample was positive for *C. orientalis* in three independent 10 µm section DNA extractions and qPCR analyses. A mean of 10.35 *C. orientalis* ITS2 rDNA copies was calculated, indicating a presence of the blood fluke species in SBT dating back to 1995.

4.6. Discussion

As far as we are aware this study describes the earliest detection of *C. orientalis* in SBT. This blood fluke species was described in 2010 (Ogawa et al., 2010), and the presence of *C. orientalis* was documented in ranched SBT serum samples from 2008 (Polinski et al., 2013a). To date, no retrospective examination for *C. orientalis* has been conducted using archival ABT and PBT samples. In three separate DNA extractions from the same FFPE block with subsequent qPCR analysis, we have identified and confirmed the presence of *C. orientalis* in ranched SBT in 1995, 15 years prior to species description from PBT in 2010 (Ogawa et al., 2010).

The application of newly developed qPCR detection methods on archival FFPE SBT samples contributes to our ability to examine and understand the historical prevalence of blood flukes in SBT. Consequently, optimization of DNA extraction is a vital preliminary step to ensure the most efficient use of archival samples. Proteinase K incubation for 72 h at 37°C provided a significant increase in DNA yield compared to 24 h. Extended incubation FFPE of samples during DNA extraction has been documented in previous studies, where 3-day proteinase K incubation has significantly increased yield of high molecular weight DNA (Gilbert et al., 2007, Cao et al., 2003, J. Isola, 1994, Shi et al., 2002). In addition to extended incubation time, exposing the sample to 90°C for 1 h resulted in a 100-fold increase in target DNA yield when compared to the same method without this step. This result suggests that the 1 h at 90°C increased total DNA yield via the reversal of formalin induced crosslinking of DNA.

Although not the most efficient DNA extraction method examined in this study, the QIAamp FFPE® kit protocol also utilizes a 90°C 1 h step and was significantly more efficient to extraction methods without the 1 h at 90°C. Silica column based DNA extraction and purification can be subject to losses of final DNA yield, though Qiagen claims this only occurs

when using fragments ranging between 70 bp and 4 kb where the estimated loss would be 20% (Qiagen, 2016). A study examining the efficacy of the Qiagen MinElute PCR Purification Kit using different-sized fragments ranging between 106 and 409 bp found a mean loss of 39.03% with no clear relationship between DNA strand length and retention (Kemp et al., 2014). Studies comparing commercial FFPE DNA extraction kits showed the most efficient protocols apply high temperature incubation (Muñoz-Cadavid et al., 2010, Shi et al., 2002). Efficient DNA extraction from archival FFPE material is challenging as the documentation of fixative pH, strength, and temperature and fixation time for the FFPE material is often unknown, which in turn makes it difficult to predict the degree of DNA degradation and cross-linking (Shi et al., 2002). In this study 1 h incubation at 90°C post proteinase K incubation has shown to significantly increase DNA yield. Other studies have reported increased DNA yield from FFPE material via the application of heat, by means of microwave, oven, or heat plate as well as alkali environments (Campos and Gilbert, 2012, Gilbert et al., 2007, Muñoz-Cadavid et al., 2010, Wu et al., 2012).

The TRIzol-based FFPE DNA extraction was among the lower DNA yielding of the 5 methods. This result differs from similar studies, which have shown superior FFPE extraction efficiency using a phenol-chloroform protocol in liver and buccal cells (Farrugia et al., 2010, Cao et al., 2003). The frequent change of tubes, and potential DNA loss during isolation of layers during phenol-chloroform based FFPE DNA extraction could have resulted in the lower target analyte yield in comparison to methods 3 and 4. The present study focused on FFPE SBT heart and gill, the organ is doubtfully the cause of variable protocol efficiency as all samples were equally homogenised proceeding with DNA precipitation.

Archival FFPE samples are a highly valuable and often limited resource allowing for retrospective prevalence and epidemiological studies. Therefore maximizing target DNA yield using an appropriate extraction protocol is of utmost importance. This study displayed the importance of extended Proteinase K incubation in addition to possible reversal of formalin induced crosslinking of DNA via a 1 h 90°C incubation step.

5. Evaluation of bacterial diversity in spleen of Southern bluefin tuna (*Thunnus maccoyii*) using 16S rRNA bacterial pyrosequencing

5.1. Introduction

In recent years researchers have increasingly explored the link between adverse health conditions and localized characterization of microbiota. More specifically, this has been investigated for human joints and bones (Jiang et al., 2015), breasts (Urbaniak et al., 2014), stomach (Bik et al., 2006), lungs (Beck et al., 2012), bladder (Wolfe et al., 2012) and blood (Amar et al., 2011, Mandal et al., 2016). Microbial imbalances in certain tissues have been associated with diabetes (Kriegel et al., 2011), inflammatory bowel disease (IBD) (Garrett et al., 2010), obesity (Turnbaugh et al., 2006), functional bowel disease (Tana et al., 2010), cardiovascular disease (Amar et al., 2013), non-alcoholic fatty liver disease (NASHD) (Abu-Shanab and Quigley, 2010), colorectal carcinoma (Castellarin et al., 2012), and psoriasis (Gao et al., 2008).

The presence of indigenous microbiota and the manipulation thereof in the digestive tract, skin, gills and other internal organs have shown to directly affect the health and disease in fish (Llewellyn et al., 2015). Additionally, the teleost gut microbiome has posed the potential to function as a biomarker for stress and disease (Llewellyn et al., 2015). In fish, species from more than 50 bacterial genera can cause disease (Austin and Austin, 2007), and without bacterial species specific infection symptoms the only method of diagnosis lies in culture/sequencing (Toranzo et al., 2005, Salén et al., 1995). The kidney is the most commonly used for the isolation of bacteria causing systemic infections (Austin and Austin, 2007). As with humans, the blood and internal organs of fish were believed to be sterile in healthy fish (Cahill, 1990, Horsley, 1977), however an increasing body of evidence documents the presence of bacteria in the blood and organs of apparently healthy fish (Table 7).

Table 7 Studies documenting bacteria in blood and/or internal organs of apparently healthy fish

Species	Number of Fish	Sample type	Method	Bacteria Identified	Percentage fish positive	Source
Silver hake (*Merluccius bilinearis*) **Squirrel hake (*Urophycis chuss*)**	3	Blood (heart)	Culture	N/A	33.3%	(Proctor and Nickerson, 1935)
Perch (*Perca flubiatilis*)	N/A	Blood (heart)	Culture	Gram-negative bacilli; Grampositive cocci; non-sporing Gram-positive bacilli	N/A	(Bisset, 1948)
Brook trout (*Salvelinus fontinalis*) Rainbow trout (*Oncorhynchus mykiss*) **Brown trout (*Salmo trutta*)** **Lake trout (*Salvelinus namaycush*)**	350	Heart, liver, kidney	Culture	*Pseudomonas, Aeromonas, Micrococcus, Lactobacillus, Escherichia , Brevibacterium, Paracolobactrum, Aerobacter, Proteus, Alcaligenes, Bacillus, Achromobacter, Flavobacterium , Streptococcus*	59.5%	(Evelyn and McDermott, 1961)
Brook trout (*Salvelinus fontinalis*) Rainbow trout (*Oncorhynchus mykiss*) **Brown trout (*Salmo trutta*)**	244	Blood (heart), kidney	Culture, enrichment	Oxidative fluorescent pseudomonads, non-oxidative, non-fluorescent pseudomonads, Flavobacteria, *Aeromonas salmonicida, A. liquifaciens*, Gram-positive rods, lactose fermenting enteric bacteria, unidentified Gram-negative rods	12.5% -26.1% (kidney) 27.5% (blood)	(Bullock and Snieszko, 1969)
White perch (*Roccus americanus*)	52	Blood (heart), liver, spleen, kidney	Culture	*Bacillus, Achromobacter, Pseudomona, Aeromonas, Enterobacter, Micrococcus, Pseudomonas*-like.	N/A	(Norris and Pelczar, 1967)
Turbot (Scophthalmus maximus)	900	Kidney, liver	Culture	*Vibrio, Pseudomonas, Aeromonas, Moraxella, Acinetobacter, Streptococcus, Staphylococcus, Micrococcus, Sarcina, Corynebacterium*	27-42%	(Toranzo et al., 1993)
Red snapper (*Lutjanus campechanus*)	60	Kidney	Culture	*Photobacterium, Vibrio, Stenotrophomonas, Enterobacter, Bacillus, Exiguobacterium, Shewanella, Pseudoalteromonas, Pseudomonas, Staphylococcus, Microbacterium*	25%	(Arias et al., 2013)
Red snapper (*Lutjanus campechanus*)	10	Blood	Culture	*Pseudomonas, Nevskia, Ralstonia, Herbaspirillum, Aquabacterium,*	100%	(Larsen, 2014)

				Alicycliphilus, Acidovorax, Methylobacterium, Methylibium, Stenotrophomonas, Corynebacterium, Micrococcus, Staphylococcus, Cetobacterium		
Elasmobranchs (Sharks and stingrays)	195	Blood (caudal vein)	Culture, enrichment	*Photobacterium, Staphylococcus, Vibrio, Pseudomonas, Pasteurella , Shewanella , Citrobacter, Stenotrophomonas, Aeromonas, Alcaligenes, Chryseomonas, Moraxella, Morganella , Plesiomonas, Proteus, Sphingomonas, Streptococcus*	26.7%	(Mylniczenko et al., 2007)

There is a discrepancy between bacterial diversity results obtained using culture-based methods and pyrosequencing results (Bahrani-Mougeot et al., 2008, Benítez-Páez et al., 2013, Janda and Abbott, 2007). Bacteria are typically identified via sequencing by targeting the 16S rRNA gene. While present in most bacteria (Janda and Abbott, 2007), this housekeeping gene contains hyper-variable regions allowing for differentiation and specific identification using high-throughput next-generation pyrosequencing. This relatively new technology is able to sequence and identify both culturable and unculturable bacteria as target genes are extracted directly from source, even from low biomass environments (Arp et al., 2013).

The physiological thermoregulatory nature of bluefin tuna allows the immune response to operate independently of ambient water temperatures (Graham and Dickson, 2004, Janda and Abbott, 2007). This may contribute to the fish's resilience to bacterial diseases (Munday et al., 2003). Worldwide only two cases of severe mortalities resulting from bacterial infections have been reported in tuna farming. The first bacterial outbreak resulting from *Photobacterium damselae* subsp. *piscicida*, the causative agent of *pasteurellosis* occurred in ABT cultured in the Adriatic Sea (Mladineo et al., 2006). During this outbreak, fish showed no signs of clinical illness apart from atypical swimming and changed coloration, this lead to the death of more than 2500 tuna within 2 days (Mladineo et al., 2006). Though researchers believe tuna are able to carry this bacteria without clinical signs of infection (Munday et al., 2003), this particular disease outbreak may have been as a result of higher pathogenicity of the particular strain or immunocompromised fish, possibly a combination of both (Mladineo et al., 2006). The second bacterial outbreak has been documented in hatchery reared ABT larvae in Italy, opportunistic

bacteria *Photobacterium damselae* subsp. *damselae*, a *Vibrio* sp. and a *Tenacibaculum* spp. were identified (Austin and Austin, 2007).

A recent culture-based study evaluated the microbial characteristics of internal and external organs of ABT noting a higher bacterial load in the gills and skin as compared to the internal organs (Kapetanović et al., 2006). While bacteria from the genera *Pasteurella*, *Brevundimonas*, *Moraxella*, *Staphylococcus*, *Vibrio*, *Klebsiella*, *Pseudomonas*, and *Weeksella* were found in the internal organs of ABT, their quantitative composition did not vary between the organs (Kapetanović et al., 2006).

Previous studies have examined the microbiological diversity of SBT using culture methods (Valdenegro-Vaga et al., 2013, Munday and Hallegraeff, 1998, Munday et al., 2003). To date *Vibrio* sp. and *Tenacibaculum* spp. have not been identified in SBT. *Photobacterium damselae* subsp. *damselae* has been documented in SBT (Valdenegro-Vaga et al., 2013) spleen and kidney, and has been shown to cause systemic infections in several other fish species (Gauger et al., 2006, González et al., 2004, Osorio et al., 2000, Pedersen et al., 2008). In addition, external lesions linked to septicaemia have been documented in the gills, skin, muscle, and internal organs of fish (Austin and Austin, 2007). Although a range of potentially pathogenic bacteria have been identified in SBT using culture methods, they have yet to be associated with disease. With a growing body of evidence documenting the presence of indigenous microbiota in previously thought to be sterile environments, this study aims to identify bacterial diversity in wild and ranched SBT spleen using barcoded 16S gene rDNA pyrosequencing.

5.2. Materials and methods

5.2.1. Ethics statement

All wild SBT sampling procedures have been approved by the University of Tasmania Animal Ethics Committee (A0013175).

5.2.2. Sampling and DNA extraction

Spleen samples of ranched SBT (n = 10) were collected alongside 2014 commercial harvest in Port Lincoln, South Australia. Wild SBT were caught between January and May 2014 (n = 10) using the trolling method in waters surrounding Pedra Branca, Tasmania. Heart and gill of the SBT utilized in this chapter have also been examined for *C. forsteri* and *C. orientalis* as described in Chapter 2.

Spleens (n = 20) were placed in individual sealed bags as soon as they were dissected from the fish and stored on ice until laboratory processing 3 hours later. A 2 cm^2 area of the spleen exterior was disinfected with a heated scalpel blade. An incision was made after re-disinfecting the blade and a 0.5 cm^3 piece of spleen was preserved in 1 mL RNA preservation reagent (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA, pH 5.2) and placed on ice for subsequent DNA extraction. DNA extraction was performed as described in Chapter 2, the pellet was suspended in 50 µL buffered water (0.05% TX-100, 10 mM Tris, pH 7.5). 4 negative extraction controls were used alongside sample DNA extraction to confirm lack of contamination via reagents.

5.2.3. PCR amplifications for pyrosequencing

The total primary PCR reaction volume was 20 µL, and consisted of 10 µL 2 x MyTaq HS mix, and 0.6 µL of 10 µM 27F (5' - GAGTTTGATCMTGGCTCAG - 3') and untagged 518R (5' - WTTACCGCGGCTGCTGG – 3') 16S rDNA gene primers, 4.8 µL buffered water (0.05% TX-100, 10 mM Tris, pH 7.5) and 4 µL of 1:10 diluted TNA extract. The PCR was performed on a C1000™ Thermal Cycler (Biorad Laboratories Inc., USA). Cycling conditions comprised an activation of DNA polymerase at 95 °C for 3 min, followed by 33 cycles of denaturation for 10 s at 95 °C, annealing for 30 s at 58 °C, followed by extension for 15 s at 72 °C. PCR products were examined on a 1.5% agarose gel. No amplification in DNA extraction controls confirmed. Gel DNA extraction of each target amplicon was conducted using the QIAquick Gel Extraction Kit® (QIAGEN) and used as per manufacturer instructions. The secondary PCR consisted of 25 cycles of the previous primer concentrations and cycling conditions, where a sequencing adapter was added to the 5' end of each reverse primer containing a sample specific barcode.

Next PCR products were purified using SureClean (Bioline Pty. Ltd., NSW, Australia) as per manufacturer instructions, and concentration of resultant extract was measured using a Qubit™ flourometer (Invitrogen™, Life Technologies Australia Pty. Ltd., Victoria, Australia). All 20 purified PCR extracts were pooled into one tube to contain 25 ng of each sample. This was purified using SureClean and quantified using Qubit. A 100 µL suspension containing 2 ng/µL of amplicon was sent to Macrogen Inc. (Seoul, Korea) for pyrosequencing (454 GS FLX, Roche, USA).

5.2.4. Pyrosequencing data analysis

After sequencing, failed sequence reads and low-quality sequence ends, barcodes, and primers were removed using Geneious R7 (Biomatters Limited). Each samples separately processed sequence FASTA file was uploaded to a Virtual Machine (VM) running CloVR (Angiuoli et al., 2011) in the Institute for Genome Sciences Data Intensive Academic Grid (DIAG) cloud-computing infrastructure. The CloVR 16S pipeline was used to conduct automated RNA sequence analysis using phylogenetic protocols and tools, more specifically: QIIME, UCHIME, Mothur, and Metastats. These tools performed automated sequence processing, phylogenetic analysis, identified 16S sequence fragments, and produced graphical visualization and statistical analysis of the data.

In the present study the automated 16S sequence analysis was included mean abundance calculations. Beta diversity refers to the measurement of varying bacterial diversity between treatments; in this case the comparison is wild versus ranched SBT spleen. Alpha diversity refers to the measurement of diversity within a community, this can be measured using different metrics, and each devised to focus on different aspects of the community structure. The operational taxonomic units (OTU) were defined with 1% dissimilarity. The coverage percentages (Good), richness estimators (ACE, Chao, Jackknife) and diversity indices were calculated using Good's method (Good, 1953) and the DOTUR program (Schloss and Handelsman, 2005).

5.3. Results

5.3.1. Summary of pyrosequencing

Pyrosequencing of produced 156,862 total reads ranging between 40 and 1068 bp in length with the mean length of 394 bp. This wide range suggests the presence of chimeric sequences due to abnormal PCR amplification or incorrect assembly.

5.3.2. Taxonomy assignment

The SBT spleen microbiota were dominated by *Proteobacteria* (94.41% ±0.041) followed by *Acidobacteria* (4.71% ±0.035), *Cyanobacteria* (0.49% ±0.007), and *Firmicutes* (0.24% ±0.005), which made up 99.85% of phyla (Figure 12). Other minor abundant phyla included *Bacteroidetes, Spirochaetes, Gemmatimonadetes, Verrucomicrobia, Armatimonadetes*, and *Planctomycetes*. *Firmicutes* did not show significant difference in mean abundance ($n = 20$) while other phyla were significantly different in mean abundance in wild and ranched SBT ($p < 0.05$). *Cyanobacteria* were more abundant in wild SBT, whereas *Acidobacteria* were significantly more abundant in ranched SBT spleen ($p < 0.05$). *Bacteroidetes* was found in wild SBT ($p < 0.0001$), but not detected in ranched samples. There was no significant difference in abundance of *Proteobacteria* in wild and ranched SBT spleen.

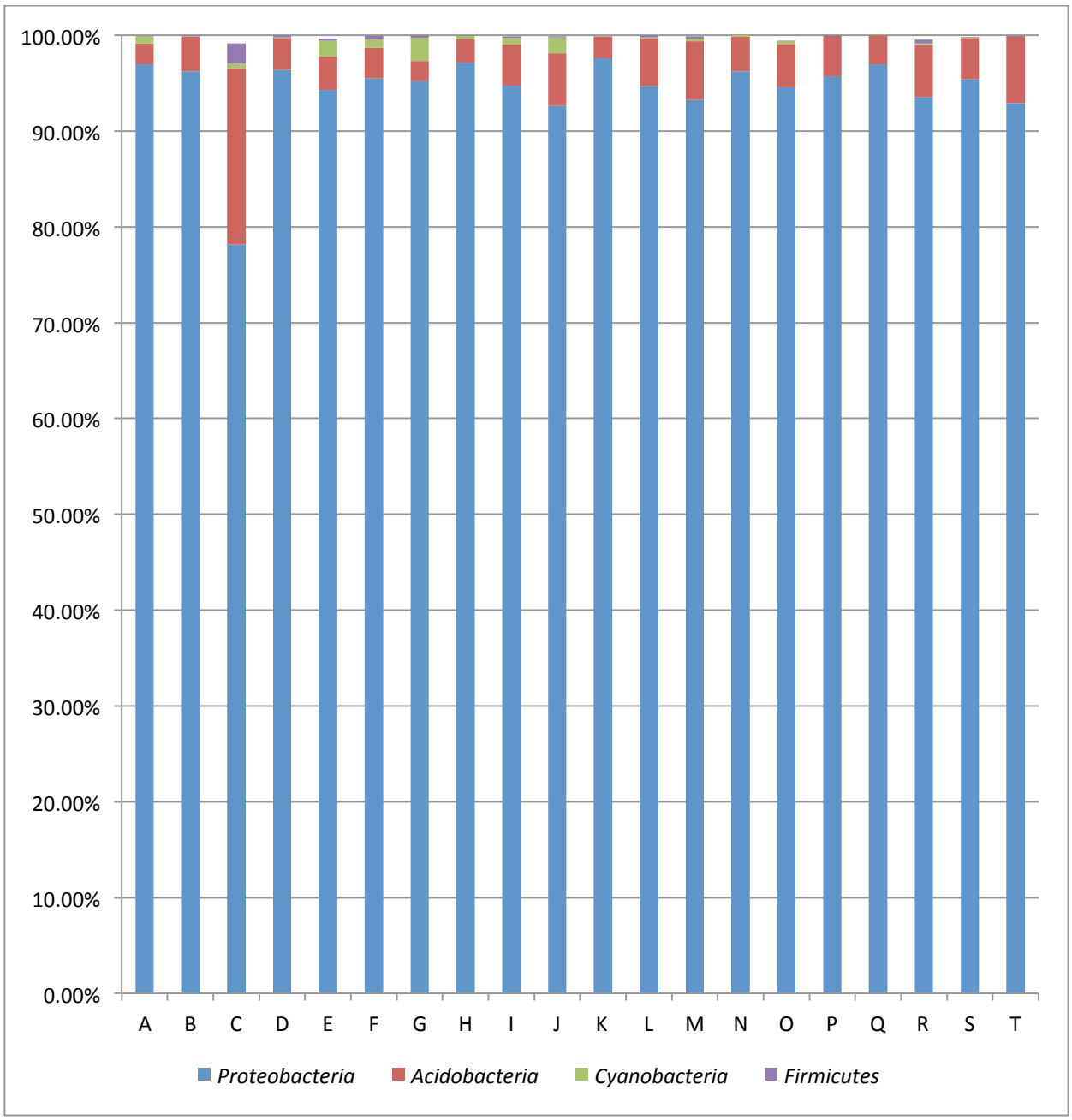

Figure 12 Stacked area graph showing abundance of top 4 phyla in wild (A-J) and ranched (K-T) SBT spleen

With ranched and wild fish grouped, top 18 most abundant genera constituted 99.2% of the spleen microbiota (Figure 13). These included *Bosea* (66.99%), *Phyllobacterium* (22.73%), *Edaphobacter* (2.74%), *Methylobacterium* (2.03%), *Propionibacterium* (2%), *Bradyrhyzobium* 0.69%), *Ochrobactrum* 0.52%), *Mesorhizobium* (0.31%), *Pseudoalteromonas* (0.21%),

Corynebacterium (0.19%), *Acinetobacter* (0.19%), *Chelatococcus* (0.14%), *Burkholderia* (0.1%), *Staphylococcus* (0.1%), *Psychrobacter* (0.08%), *Yersinia* (0.06%), *Enterobacter* (0.06%), and *Pseudomonas* (0.05%). Although *Edaphobacter* and *Pseudoalteromonas were* present in both groups, they were significantly more abundant in ranched SBT spleen ($p < 0.05$). Interestingly, 6 reads of *Staphylococcus* were documented in a single wild SBT spleen while it was not shown in ranched SBT spleen samples, making it significantly more prevalent in wild SBT spleen.

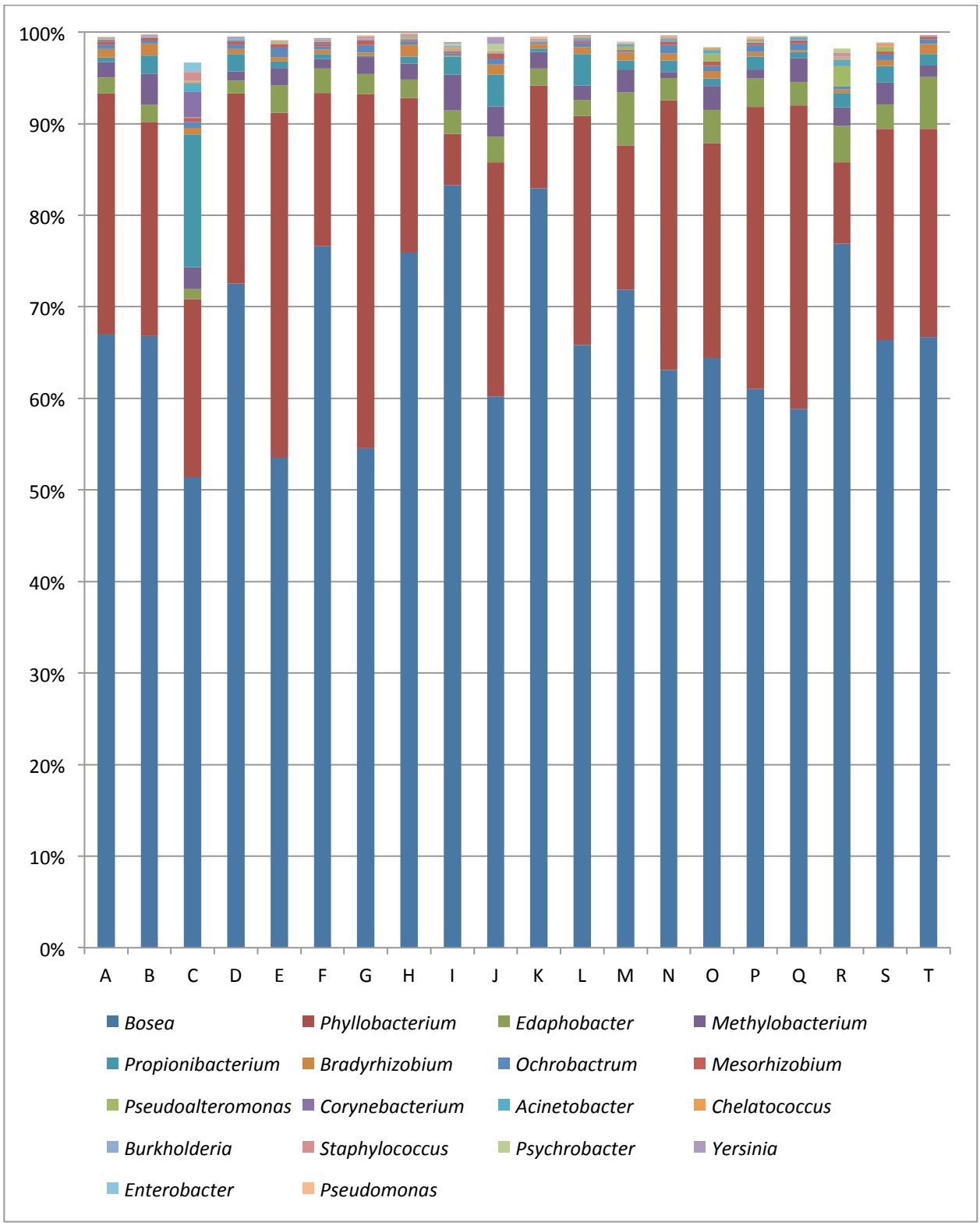

Figure 13 Stacked area graph showing abundance of top 18 genera in wild (A-J) and ranched (K-T) SBT spleen

5.3.3. Alpha diversity analysis

On average the SBT spleen (wild and ranched samples grouped) microbiota had 148.9 (±35.4) OTUs (min = 109; max = 252) at 97% sequence similarity (Figure 14) (Table 8). No significant difference was found among any of the tested indices.

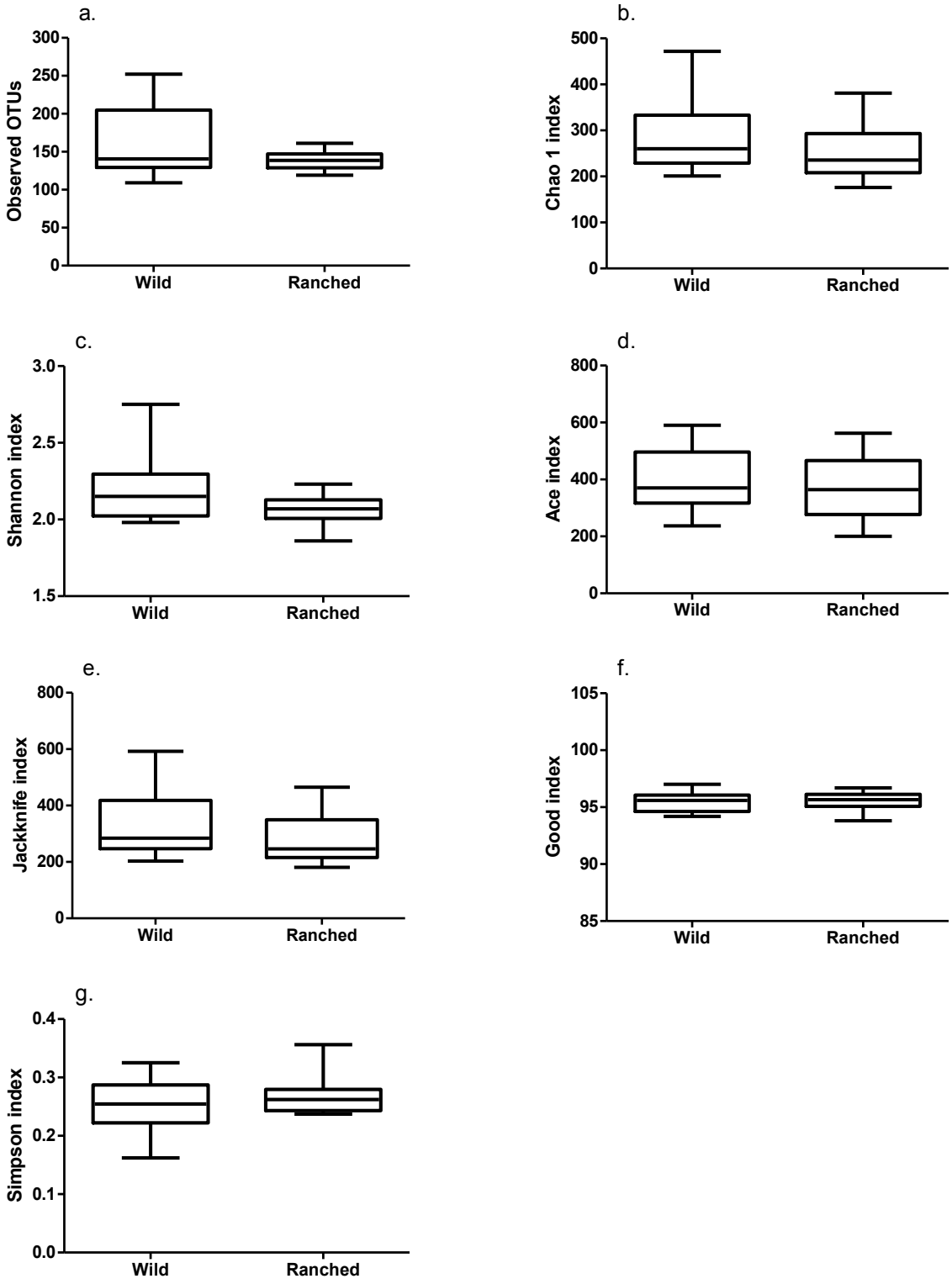

Figure 14 Alpha diversity index of microbiota OTU table. Boxes display the 75% Cl and line the mean. Whiskers display the minimum and maximum values.

Table 8 Phylotype and diversity estimation of the 16S rRNA gene libraries from the pyrosequencing analysis

	Sample	Reads	OTU[a]	Good[b]	Chao	ACE	Jackknife	Shannon	Simpson
Wild SBT	A	6135	216	96.5	366	509	415	2.20	0.254
	B	2455	109	95.6	207	326	223	2.09	0.255
	C	3453	201	94.2	282	290	287	2.75	0.162
	D	3058	134	95.6	253	365	300	2.03	0.279
	E	3334	147	95.6	267	425	281	2.24	0.230
	F	8296	252	97.0	472	590	592	2.15	0.288
	G	2263	121	94.7	244	375	272	2.15	0.238
	H	3245	132	95.9	236	346	255	2.00	0.287
	I	2766	132	95.2	201	237	203	1.98	0.325
	J	2771	156	94.4	322	492	427	2.46	0.198
Ranched SBT	K	2428	119	95.1	211	352	233	1.86	0.356
	L	3627	140	96.1	232	248	259	2.01	0.273
	M	3496	138	96.1	221	398	220	2.06	0.273
	N	3136	150	95.2	344	528	426	2.21	0.237
	O	1928	119	93.8	176	200	181	2.23	0.238
	P	2957	139	95.3	276	446	324	2.10	0.247
	Q	3389	134	96.0	269	351	321	2.05	0.245
	R	2654	132	95.0	200	287	203	2.10	0.299
	S	4289	161	96.2	381	562	465	2.08	0.257
	T	4405	146	96.7	239	376	227	2.00	0.267

5.3.4. Beta diversity analysis

Principal coordinate analysis (PCoA) plot based on weighted UniFrac distance showed no overall difference between wild and ranched SBT spleen in the community structure. Apart from one data point in the wild group, which was distinct from the rest of the community, the wild and ranched groups seemed to be of equal dissimilarity (Figure 15). When observing the PCoA plot based on unweighted UniFrac distance, one could stipulate a similarity between wild data points compared to the ranched community structure (Figure 16).

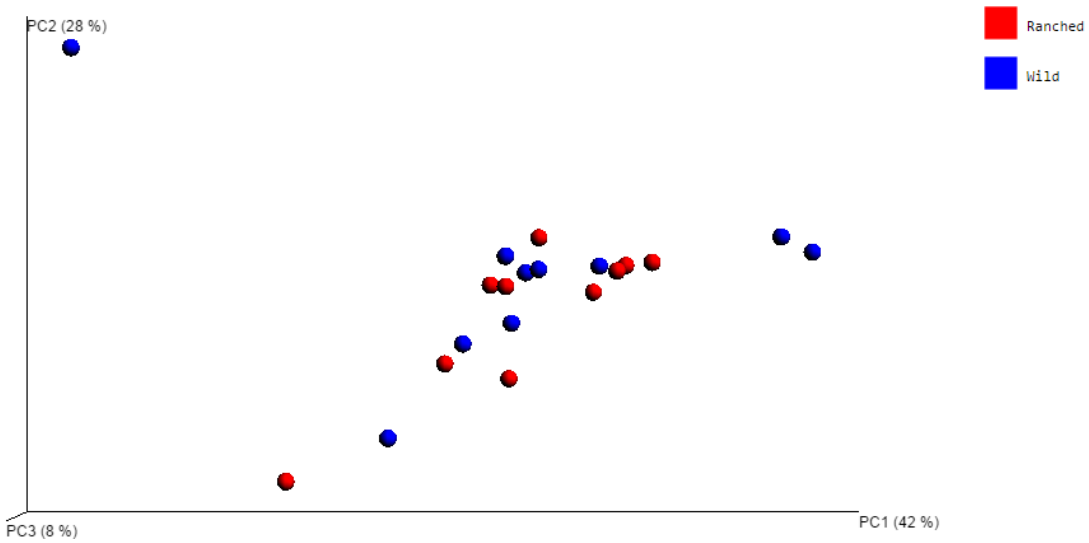

Figure 15 Principal Coordinate Analysis (PCoA) plot of SBT spleen microbiomes. PCoA plot of wild (n = 10) and ranched (n = 10) SBT spleen using weighted UniFrac metric at even sampling depth of 8000 reads per sample.

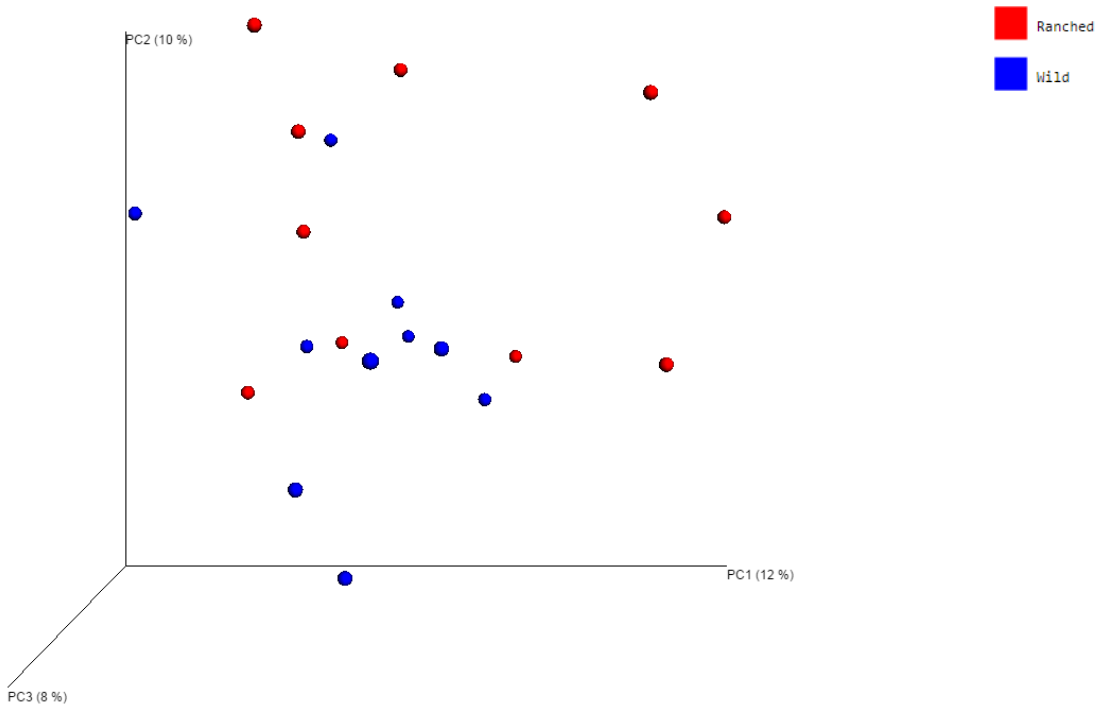

Figure 16 Principal Coordinate Analysis (PCoA) plot of SBT spleen microbiomes. PCoA plot of wild (n = 10) and ranched (n = 10) SBT spleen using unweighted UniFrac metric at even sampling depth of 8000 reads per sample.

5.4. Discussion

This study investigated the spleen microbiota of wild SBT in relation to that of ranched fish. The most abundant phylum among both wild and ranched SBT spleen was *Proteobacteria* (94%), which in turn has been documented at similar abundance in pyrosequencing analysis of the blood of red snapper (*Lutjanos bohar*) (97%) (Larsen, 2014), humans (85-90%) (Treanor and Mandell, 2000, Amar et al., 2011), and broiler chickens (60.5%) (Mandal et al., 2016). Similarly, while much less abundant, *Acidobacteria*, *Cyanobacteria*, and *Firmicutes* were also the next most abundant phyla documented in human and chicken blood (Mandal et al., 2016, Amar et al., 2011). Bacteria of the phylum *Acidobacteria* have previously been documented in freshwater ponds in the Donãna National Park (Spain) (Zimmermann et al., 2012). *Cyanobacteria* has been linked to the production of geosmin and 2-methylisoborneol eutrophication in USA ponds and lakes (Van der Ploeg et al., 1992, Smith, 2003).

Bacterial species belonging to the phylum *Firmicutes* have been documented in the skin, mucus, gills and gut of wild fresh water and marine fish (Smriga et al., 2010, Skrodenyte-arbaciauskiene et al., 2008, Mouchet et al., 2012, Dhanasiri et al., 2011, Deobagkar et al., 2012, Clements et al., 2007). Bacteria of the phyla *Cyanobacteria* and *Acidobacteria* were significantly more abundant in wild vs. ranched, or ranched vs. wild SBT spleen, respectively. Nevertheless, given the healthy condition of the wild and ranched fish, one could speculate that these were “indigenous” phyla. In addition, future studies may also benefit from further investigation of the microbial diversity of SBT gut.

Within the proteobacteria phylum microbiota from the genus *Bosea* were most abundant in both wild and ranched SBT spleen. Species from this genus have not been reported from fish, having been primarily documented to occur naturally in fresh water, such as in hospital intensive care

supplies (La Scola et al., 2003b, La Scola et al., 2003a) and general drinking water (Lee et al., 2010, Thomas et al., 2007). In the hospital environment *Bosea* species have been linked to ventilator-associated pneumonia in immunocompromised patients (La Scola et al., 2003a, Kakizaki et al., 2012). Hence, one could speculate that the abundance of *Bosea* in SBT spleen may be "indigenous", and may opportunistically cause disease when the fish is stressed/immunocompromised.

Microbiota from the genus *Phyllobacterium* and *Edaphobacter* of the phylum *Proteobacteria* had the 2nd and 3rd highest abundance respectively and have both been documented in marine environment. *Phyllobacterium* species are frequently detected in the marine water column (Hwang and Cho, 2008) and it has also been found in the digestive tract of sea urchins (Meziti et al., 2007). Similarly, genus *Edaphobacter* is fresh-water environment-based, having been documented in Alaskan glaciers (Segawa et al., 2010). The pathogenic nature of *Methylobacterium* has been attributed to bacteremia in human immunocompromised patients (Hornei et al., 1999), primarily due to contaminated hospital equipment (Imbert et al., 2005, Li et al., 2015). This genus has been found in air as well as freshwater aquatic environments and is not associated with pathogenicity (Hiraishi et al., 1995, Dash et al., 2013). In addition, the abundance of *Propionibacteria* was surprising; this genus of bacteria has not been detected in fish and is found in sweat glands, it has been stipulated to be the most prevalent microorganism found in human skin (Brüggemann et al., 2004, Leyden et al., 1983). Hence, the abundance of *Phyllobacterium Edaphobacter, Methylobacterium* and *Propionibacteria* in SBT spleen may represent contamination.

Water-borne bacteria such as *Pseudomonas*, *Stenotrophonas*, *Xanthomonas*, *Ralstonia* and *Bacillus* have been found in molecular-grade laboratory reagents and can lead to artificial

inflation of diversity estimates in 16S pyrosequencing microbiome studies (Grahn et al., 2003, Salter et al., 2014, Kunin et al., 2010). Particularly in low biomass environments, resultant species composition of PCR-based 16S rRNA gene surveys and metagenomics analyses has shown to vary significantly between DNA extraction kits, reagents, commercial kits and kit batches (Salter et al., 2014). As applied in the present study, the influence of contamination can be minimized with rigorous quality based trimming of 16S pyrotags and the use of clustering thresholds no lower than 97% identity (Kunin et al., 2010).

Pseudoalteromonas was among the 18 most abundant genera presented in this study. Species of this genus have been associated with red spot disease in *Laminaria japonica* sea weed (Sawabe et al., 1998), and have been linked to marine eukaryotes where they have shown anti-bacterial, bacteriolytic and algicidal properties (Holmström and Kjelleberg, 1999). In addition, a recent study succeeded in isolating this same genus using culture-methods from the intestine of reared and healthy sea bass (*Dicentrarchus labrax*) and effectively used it as a probiotic (Mladineo et al., 2016). Interestingly, this is the only genus also detected in a previous culture-based microbiological study of SBT spleen (Valdenegro-Vaga et al., 2013). The reason for other bacteria being differentially detected was application of culture methods in the previous study rather than 16S pyrosequencing used here. In fact, various studies have explored this subject and have shown the bacterial diversity obtained using culture methods is drastically lower than when using pyrosequencing (Benítez-Páez et al., 2013). This discrepancy remains, even when culture conditions entail a range of media in both aerobic and anaerobic culture conditions typically applied to expand the array of microorganisms (Bahrani-Mougeot et al., 2008). More specifically, a study examining transient bacteremia following dental extraction in humans found that culture methods produced 1-2 isolates, whereas pyrosequencing indicated the mean

abundance of 22.8 (±1.1) different bacteria genera (Benítez-Páez et al., 2013). Hence, this study may present a more comprehensive overview of bacterial diversity in SBT spleen compared to preceding culture-based studies.

Pseudomonas, Enterobacter, Staphylococcus were not the most abundant genera in the present study, however this was the case in a pyrosequencing examination of apparently healthy red snapper blood (Larsen, 2014). Other culture-based studies (see Table 7) identifying genera found in the blood and internal organs of healthy marine and fresh water fish have documented *Stenotrophomonas, Photobacterium* (Arias et al., 2013, Mylniczenko et al., 2007), *Vibrio, Staphylococcus* (Arias et al., 2013, Mylniczenko et al., 2007, Toranzo et al., 1993), *Achromobacter* (Norris and Pelczar, 1967, Evelyn and McDermott, 1961)*, Bacillus* (Norris and Pelczar, 1967, Arias et al., 2013, Evelyn and McDermott, 1961), *Streptococcus* (Mylniczenko et al., 2007, Toranzo et al., 1993, Evelyn and McDermott, 1961), *Micrococcus* (Toranzo et al., 1993, Evelyn and McDermott, 1961), *Enterobacter* (Norris and Pelczar, 1967, Arias et al., 2013), and *Aeromonas* (Bullock and Snieszko, 1969, Norris and Pelczar, 1967, Mylniczenko et al., 2007, Toranzo et al., 1993, Evelyn and McDermott, 1961). Although increased abundance of bacteria in blood of turbot with external clinical signs of disease have been documented, no association of bacterial species to specific pathology has been shown (Toranzo et al., 1993).

When examining variability in abundance among individual red snapper gill, feces and blood, the latter showed the least variability between individuals with 58% similarity, compared to 22.4% and 21.3% respectively. Bacterial communities consisting of 148 (±35.4) OTUs were present in the SBT spleen, irrespective of whether from wild or ranched SBT with 97% similarity in species abundance. Beta diversity analysis showed no distinct difference in bacterial communities between wild and ranched fish, suggesting the documented diversity may represent

the baseline. This study has added to the growing body of evidence that fish blood can contain a high bacterial diversity without showing external signs of disease.

6. General Discussion

Hydrolysis probe-based qPCR assays are already widely used for the early pathogen detection and diagnosis in other industries, whether it be the detection of *Enterobacter sakazokii* in infant formula (Liu et al., 2006) or *Brugia malayi* and *B. timori* DNA in human blood (Rao et al., 2006). This research presents a new hydrolysis probe-based quantitative assay for detection of 3 species from the genus *Cardicola*, where *C. forsteri* and *C. orientalis* are known to infect SBT (Polinski et al., 2013a). Though previous studies have developed qPCR-based quantification of *Cardicola* in SBT, these methods utilized DNA intercalating SYBR green chemistry which, to ensure maximum sensitivity and specificity, requires expert interpretation of melt curve analysis at target analyte concentrations nearing the assay LOQ and LOD (Polinski et al., 2013a). Moreover, the TaqMan CR approach described in the previous study was advised to be used in conjunction with SYBR and subsequent melt-curve analysis due to risk of assay inhibition when high levels of gDNA were present (Polinski et al., 2013a). The ease of use, accuracy and sensitivity of the assay described and validated in this thesis facilitates early *C. forsteri* and *C. orientalis* detection in ranched SBT.

The SBT industry has reported <1% fish mortality since the introduction of PZQ (Polinski et al., 2014), yet the present study showed up to 98% *C. forsteri* infection in SBT heart samples. This suggests that increased SBT infection with *C. orientalis* infection but not with *C. forsteri* prior to 2013 may be linked to the increased mortalities. Regardless of the peak *C. forsteri* infection prevalence of 100% in ranched SBT within two months of transfer to sea cages followed by a decline in prevalence to 35% (Aiken et al., 2006), the fish condition index remained consistent, leading Aiken et al. to conclude that SBT are not affected by *C. forsteri* infection.

The significantly improved DNA extraction efficacy from paraffin blocks achieved by the incorporation of 90°C 1 h incubation step led to the earliest detection of *C. orientalis* in SBT, 15 years prior to species description in 2010 (Ogawa et al., 2010). Although a retrospective study documented *C. orientalis* in SBT serum in 2008 (Polinski et al., 2013a), the present study indicates that the blood fluke species has been prevalent in ranched SBT even longer. C. *forsteri*, *C. orientalis*, and *C. opisthorchis* infections have been documented in ranched and wild ABT and PBT (Forte-Gil et al., 2016, Sugihara et al., 2016, Aiken et al., 2007b). *C. opisthorchis* has not been documented in SBT, including in this study. In addition, the FFPE DNA extraction method described here was significantly more efficient than the QIAamp FFPE® kit, which has proven to be the among the best in a similar study comparing commercial kits for FFPE DNA extraction (Farrugia et al., 2010).

Both the presented FFPE DNA extraction method and the QIAamp FFPE® kit encompass a step in which the sample is exposed to 90°C for an hour. The QIAamp FFPE® kit utilized silica columns, which can be subject to losses of final DNA yield, however Qiagen declares a maximum of 20% DNA loss is only seen when using DNA fragments ranging between 70 bp and 4kb (Qiagen, 2016). Conversely, a study examining the efficacy of the Qiagen MinElute PCR Purification Kit using different-sized fragments ranging between 106 and 409 bp found a mean loss of 39% with no clear relationship between DNA strand length and retention (Kemp et al., 2014). Based on the present study, single tube FFPE DNA extraction may avoid potential DNA loss presented by the use of silica columns and is therefore highly recommended for future FFPE DNA extractions.

Today real-time qPCR is frequently used to monitor viral (Miagostovich et al., 2008), bacterial and protozoan (Audemard et al., 2004, Hung and Remais, 2008) pathogens in environmental

samples including sewage (Guy et al., 2003, Lee et al., 2006, Shannon et al., 2007), farm run-off (Thurston-Enriquez et al., 2002), and in fresh/sea water (Girones et al., 2010, Hyman and Collins, 2012, Wright et al., 2015). Though this study successfully applied qPCR to detect *C. forsteri* and *C. orientalis* ITS2 rDNA in sea water (Chapter 4), future studies should explore the filtration of larger volumes of water.

Where previous studies have relied on culture methods to assess the SBT microbiome (Valdenegro-Vaga et al., 2013), this study used DNA extraction, PCR amplification, and gel DNA extraction methods during sample preparation to assess the diversity of bacteria in SBT spleen using next-generation high-throughput pyrosequencing (Chapter 5). With research increasingly documenting the presence of indigenous microbiota in organs previously thought to be sterile (Abu-Shanab and Quigley, 2010, Beck et al., 2012, Urbaniak et al., 2014, Wolfe et al., 2012), the abundance of bacteria in SBT spleen was somewhat expected. As evidenced in the present study; there was no difference in bacterial communities when comparing wild and ranched SBT. Abundance of bacteria in SBT internal organs is a relatively un-researched subject; with the increasing awareness of the link between microbial imbalances and adverse health complications (Turnbaugh et al., 2006, Amar et al., 2013, Amar et al., 2011); this research has established a baseline of microbiota identified in spleen of both wild and ranched SBT. The foundation of bacterial communities in SBT spleen presented in this study will undoubtedly contribute to future research evaluating the bacterial composition variation and effect of specific species on the health of SBT in wild and culture conditions.

6.1. Research challenges

Understanding the differences in pathogen prevalence in wild versus ranched SBT and their respective intra-annual variation is important from both conservation and ranching management. Although there have been studies concerning the prevalence of blood flukes from genus *Cardicola* in wild vs. ranched ABT (Ybañez et al., 2011, Forte-Gil et al., 2016) and PBT (Sugihara et al., 2016), none have applied molecular methods to examine tuna for multiple consecutive years. This was partially due to the limitation of examination techniques specific and sensitive enough to permit quantification and differentiation of species from the genus *Cardicola* at low levels. Previous studies in PBT and ABT have examined for presence/absence of target *Cardicola* ITS2 rDNA, rather than DNA quantification (Shirakashi et al., 2013, Ogawa et al., 2011, Forte-Gil et al., 2016). This obstacle was overcome in this thesis by providing key information based on hydrolysis probe-based qPCR detection and quantification of *Cardicola* in SBT heart and gill, formalin-fixed paraffin-embedded archival samples, and DNA in water.

Completing this research in a close collaboration with commercial ranching industry presented some challenges. Sampling of harvest fish was conducted opportunistically alongside routine harvest operations. In 2014 sampling was delayed due to weather conditions, which resulted in reduced time to collect; process and ship samples back to Launceston. Acquisition of wild SBT samples around the Tasman Peninsula and Pedra Branca was challenging, the costs of hiring charter boats, weather conditions, absence of fish in the area and highly variable success-rate of trawling considerably affected wild SBT numbers available for the research.

Lastly, the prevention of contamination during sample preparation for 16S gene pyrosequencing was a challenge. Though quality based trimming of 16S pyrotags and the use of clustering thresholds no lower than 97% identity can minimize the impact of minor contamination on the

findings (Kunin et al., 2010), future studies would benefit from immediate, onsite sample dissection and transfer to preservation solution. The delay between sampling and sample transfer to preservation solution may have introduced contamination in the present study, even with negative controls, PCR controls and extraction controls. With cost of operation and the lack of standardized/validated protocols ruling against the popularization of molecular techniques (Girones et al., 2010), mitigation of intra-laboratory variability via publication of reproducible methods would facilitate the widespread adoption of this technology.

6.2. Future research

Studies of *C. forsteri* treatment in SBT documented high efficacy using PZQ at various concentrations (*in vitro*: 1.5 μg/mL - 200 μg/mL, *in vivo*: 75 mg/kg - 150 mg/kg) (Hardy-Smith et al., 2012). Similarly, in juvenile PBT 7.5mg/kg treatments of once per day for 3 consecutive days achieved complete eradication of C. *opisthorchis* (Shirakashi et al., 2012a). Future studies should explore the prevalence of *C. forsteri* and *C. orientalis* throughout the ranching season and assess species-specific efficacy of PZQ in mitigating blood fluke infection in SBT. This would further elucidate changes in species prevalence of infections with blood flukes since the introduction of PZQ in 2013.

The qPCR assay described in the present study relies on laboratory resources. Future studies may explore the addition of this assay to SBT health point-of-care (POC) testing procedure at the site sampling. These methods do not require laboratory staff, require minimal introduction, and results are typically simple and do not require interpretational skills (Gubala et al., 2011). POC PCR testing in resource-limited settings have been applied to detect HIV-1 and Ebola in remote

areas, accelerating treatment and mitigating the spread of disease (Walker et al., 2015, Jangam et al., 2013). The main challenge of POC is developing a system that does not rely on operator training and is not affected environmental parameters while minimizing equipment the cost (Wang et al., 2016, Dineva et al., 2007). In SBT a POC system would allow timely treatment and prevention of financial losses due to fish mortality and hampered seasonal growth caused by parasite infection (Aiken, 2009). Depending on the number of sample analyses required the investment in a POC system could be cost-effective, as seen in large European salmon companies, which have their own PCR labs to test for specific pathogens (Nowak, 2017).

To date POC diagnostic assays can consist of paper-based assays (Wang et al., 2016, Carrilho et al., 2009, Martinez et al., 2008, Shafiee et al., 2015), microfluidics technologies (Wang et al., 2011, Wang et al., 2012, Inci et al., 2012, Wang et al., 2014), and a range of biosensors have been successfully developed and applied to detect and quantify proteins (Wang et al., 2011, De La Rica and Stevens, 2012), cells (Moon et al., 2009, Moon et al., 2011), viruses (Shafiee et al., 2013, Shafiee et al., 2014), bacteria (Wang et al., 2012, Lissandrello et al., 2014, Tokel et al., 2015) or nucleic acids (Baeumner et al., 2004) from a variety of clinical sample matrices.

In addition, given the high market value of each ranched SBT future studies could explore the development of non-lethal sampling methods. This would allow regular infection assessments throughout the season, and not exclusively at harvest as in the present study.

6.3. Conclusion

This thesis explored the application of molecular methods for the identification and quantification of pathogens in SBT and environmental samples. The methods described here

provide a set of tools that will facilitate future disease diagnosis and has given a first-time post PZQ treatment insight into variability of *C. forsteri* and *C. orientalis* prevalence in wild and ranched SBT. The specificity and versatility of the hydrolysis probe-based qPCR assays described herein lay the foundation for development of future diagnostic methodologies, be it from SBT, FFPE or sea water. The use of POC systems for rapid disease detection and prevention have already been implemented in commercial salmon production systems (Nowak, 2017). Given the monetary and environmental value of the fish, the Australian SBT ranching industry would greatly benefit from investing in a POC system for the detection of *Cardicola* spp. The understanding of molecular pathogen detection and DNA extraction methods, and evaluation of bacterial diversity in SBT attained in this thesis will undoubtedly play a significant role in ensuring a sustainable future for the species.

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